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Queen Thomas

Printed Name

Queen Thomas

Signature

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Blaszczak et al.)	
)	
Serial No.	:	09/833,647)	
)	Group Art Unit:
Filed	:	18 April 2001)	1624
)	
For	:	PROCESS FOR PREPARING LIPID II)	
)	Examiner
Docket No.	:	X-13168)	Unknown

ATTENTION: ROBERT CLARKE – LEGAL ADVISOR

RECEIVED

AUG 22 2001

PETITION UNDER 37 C.F.R. § 1.183

OFFICE OF PETITIONS

TO WAIVE REQUIREMENT

FOR CERTIFIED COPY OF FOREIGN-FILED APPLICATION

UNDER 37 C.F.R. § 1.217(c)(1)

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Attn: Box Office of Initial Patent Examination

Care of: Robert Clarke, Legal Advisor

Assistant Commissioner for Patents

Washington, D. C. 20231

Sir:

Applicants hereby respectfully petition the Commissioner for Patents under 37 C.F.R. § 1.183 to waive the requirement under 37 C.F.R. § 1.217(c)(1) for a certified copy of Applicants' PCT application PCT/US01/12637 filed April 18, 2001, which is a redacted version of above-identified U.S. application Serial Number 09/833,647. Applicants respectfully submit that they have made a good faith effort to comply with all requirements set forth in 37 C.F.R § 1.217, and that the requirement for filing of a certified copy of PCT application PCT/US01/12637 should be suspended or waived by the Commissioner or his

designee in the instant situation for the following reasons:

1. The requirement under 37 C.F.R. § 1.217(c)(1) is not a requirement of the Patent Statutes. In accordance with 37 C.F.R. § 1.183, the requirement under 37 C.F.R. § 1.217(c)(1) for a certified copy of Applicants' PCT application PCT/US01/12637, which corresponds to U.S. application Serial Number 09/833,647, for which redaction is requested in a Request submitted concurrently herewith, is not a requirement of the Patent Statutes, 35 U.S.C. Under 37 C.F.R. § 1.183, such requirement under 37 C.F.R. may be suspended or waived by the Commissioner or the Commissioner's designee on petition of the interested party, subject to other requirements that may be imposed.

2. It is impossible for Applicants to meet the deadline for filing a certified copy of PCT application PCT/US01/12637. It is impossible for Applicants to meet the deadline for filing a certified copy of PCT application PCT/US01/12637 due to a backlog in the Certification Office at the U.S.P.T.O., of which Applicants only became aware on August 8, 2001. On August 8, 2001, Ms. Tammy Pettigrew, Patent Support Staff Supervisor at Eli Lilly and Company (Lilly), contacted the Certification Office of the U.S. Patent and Trademark Office (U.S.P.T.O.) to inquire about obtaining a certified copy of PCT application PCT/US01/12637. Ms. Pettigrew was informed by Ms. Christine Hightower of the U.S.P.T.O. Certification Office that processing of certified copies of PCT applications by the U.S.P.T.O. is currently taking at least 25 to 30 days.

In an attempt to expedite this process, on August 8, 2001 Ms. Pettigrew contacted Cantwell and Paxton, Inc., 2001 Jefferson Davis Highway, Suite 1003, Arlington, VA 22202, the Washington, D.C.-based courier service routinely employed by Lilly to fulfill its urgent needs for documents and other patent-related services, including obtaining patent prosecution histories, foreign filing licenses, certified copies of patent documents, and hand-carrying PCT applications for filing in the U.S.P.T.O. Customarily, Cantwell and Paxton fills expedited requests for certified copies of patent applications within 7 (seven) days. Relying on its longstanding experience with Cantwell and Paxton, Lilly assumed that its request for a certified copy of PCT/US01/12637 would also be filled within 7 days. However, Cantwell and Paxton informed Lilly that in view of the U.S.P.T.O. backlog in providing certified copies of PCT applications, it too could not obtain the required certified copy of PCT/US01/12637 in time for filing on August 18, 2001.

In a third effort to obtain a certified copy of PCT application PCT/US01/12637 for filing by August 18, 2001, Ms. Pettigrew contacted Ms. Pat Paxton of Cantwell and Paxton on August 13, 2001 to determine if any other procedure(s) existed for obtaining a certified

copy of a PCT application in expedited fashion. Ms. Paxton referred Ms. Pettigrew to Mr. Michael Lee in the Certification Office of the U.S.P.T.O. to inquire about a procedure entitled "at cost," that facilitates obtaining certified copies of PCT applications from the U.S.P.T.O. within 72 hours for prescribed fees. Upon contacting Mr. Lee on August 13, 2001, Ms. Pettigrew was informed that since PCT applications are no longer microfilmed by the U.S.P.T.O., and unlike U.S. patent applications that are scanned, the U.S.P.T.O. Certification Office must request a physical copy of the PCT filewrapper, and could not provide a certified copy of PCT application PCT/US01/12637 in less than 17 days.

Therefore, in view of the current backlog of requests for certified copies of PCT applications at the U.S.P.T.O. Certification Office, due to the fact that there is currently no expedited procedure that will permit Lilly to obtain a certified copy of PCT application PCT/US01/12637 in less than 17 days, and although Lilly has made several good faith attempts to obtain such a certified copy in the required timeframe, it is currently impossible to obtain a certified copy of PCT application PCT/US01/12637 in time for filing by August 18, 2001. Therefore, the requirement under 37 C.F.R. § 1.217(c)(1) is impossible to meet before the deadline for requesting redaction. Applicants have requested a certified copy of PCT application PCT/US01/12637, and will forward the same to the U.S.P.T.O. as soon as it is received.

3. Grant of this Petition can create no risk to the U.S.P.T.O. of accepting inaccurate content of redacted U.S. application Serial Number 09/833,647. Applicants' PCT application PCT/US01/12637 was filed in the U.S.P.T.O. as the Receiving Office on April 18, 2001. For the convenience of the Petitions Examiner, submitted herewith is a photocopy of said PCT application as filed, as well as a photocopy of the PCT Request Form that accompanied this application filing on April 18, 2001. As a copy (the "home copy" (HC)) of PCT application PCT/US01/12637 is retained in the Receiving Office (PCT Article 12(1)), the U.S.P.T.O. is currently in possession of an official copy Applicants' PCT application PCT/US01/12637 as originally filed. Attorney for Applicants hereby certifies that the copy of the PCT application attached herewith is a true and identical copy of the application filed in the U.S. Receiving Office on April 18, 2001.

Applicants respectfully submit that grant of this Petition can create no risk to the U.S.P.T.O. of accepting inaccurate content of redacted U.S. application Serial Number 09/833,647, a problem addressed by the formality of Certification, since the U.S.P.T.O., acting as Receiving Office for PCT administration, has at all times been in full physical possession and control of PCT/US01/12637.

4. This is an extraordinary situation in which Applicants would be harmed by publication of U.S. application Serial Number 09/833,647 in unredacted form. Finally, Applicants respectfully submit that this is an extraordinary situation in which they would be harmed by publication of U.S. application Serial Number 09/833,647 in unredacted form, and requires justice in accordance with 37 C.F.R. § 1.183.

Applicants filed U.S. application Serial Number 09/833,647, for which they now seek publication in redacted form in accordance with 37 C.F.R. § 1.217, as part of a patent strategy designed to protect Eli Lilly and Company's (Lilly's) interest in, and commercial development of, a new pharmaceutical compound. Publication of U.S. application Serial Number 09/833,647 in unredacted form will jeopardize Lilly's investment of research resources and funds made in connection with discovery and development of this pharmaceutical. Further, such publication will adversely affect Lilly's future ability to commercially develop this pharmaceutical and make it available to the public. Thus, the public stands to be harmed as well by publication of Applicants' U.S. application Serial Number 09/833,647 in unredacted form.

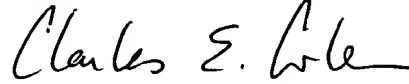
Conclusion

Therefore, for all the foregoing reasons, Applicants respectfully petition the Commissioner under 37 C.F.R. § 1.183 to suspend or waive the requirement for a certified copy of Applicants' PCT application PCT/US01/12637 under 37 C.F.R. § 1.217(c)(1) in the instant situation, and permit publication of a redacted version of Applicants' U.S. application Serial Number 09/833,647.

Please charge Deposit Account No. 05-0840, in the name of Eli Lilly and Company, in the amount of \$130.00 to cover the fee for this Petition under 37 C.F.R. § 1.17(h). Please charge any additional fees required under 37 C.F.R. 1.16 or 1.17, or credit any overpayment, to Deposit Account No. 05-0840.

If the Petitions Examiner has any questions, or would like to discuss any matter in connection with this Petition, the Examiner is invited to contact the undersigned by telephone at 317-433-4983.

Respectfully submitted,



Charles E. Cohen, Ph.D.
Attorney for Applicants
Registration No. 34,565
Phone: 317-433-4983

Eli Lilly and Company
Patent Division/CEC
Lilly Corporate Center
Indianapolis, Indiana 46285

17 August 2001

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AUG 22 2001

OFFICE OF PETITIONS

Attachments:

1. Photocopy of PCT application PCT/US01/12637 as filed on April 18, 2001;
2. Photocopy of PCT Request Form as filed on April 18, 2001 in connection with PCT application PCT/US01/12637.



-1-

**PROCESS FOR PREPARING
LIPID II**

FIELD OF THE INVENTION

5 The present invention relates to a process for
preparing substrates for the transglycosylase enzymes of
bacterial cell wall biosynthesis and the substrates produced
therefrom, in particular, the chemical synthesis of Lipid II
and analogs thereof.

10

BACKGROUND OF THE INVENTION

Potent and notably non-toxic cell wall biosynthesis
inhibitors have dominated treatment regimens for management
of bacterial infections in both hospital and outpatient
15 settings for more than fifty years. Recently, however,
resistance of bacteria to these antibiotics has reached an
alarming level and has begun to erode their once dependable
clinical efficacy. Consequently, discovery of new drugs for
the cell wall active antibacterial pharmacopoeia is urgently
20 needed.

Cell wall (peptidoglycan) biosynthetic enzymes and
their natural substrates can be very useful tools in studies
directed to understanding resistance mechanisms at the
molecular level or to the search for newer and more
25 effective agents. Although the transpeptidase enzymes are
well-known to be targets of the β -lactam antibiotics,

studies on the transglycosylation process have been limited primarily due to poor accessibility of the natural transglycosylation substrate, Lipid II and its analogs. Isolation of Lipid II from liquid cultures of bacteria presents several formidable challenges, the most obvious being scale. The level of Lipid II has been estimated to be only 1,000 - 2,000 molecules per cell in *Escherichia coli*. Fifteen hundred molecules per cell equates to approximately 2.5 nM in a dense (10^9 cfu/ml) culture. Although 50 μ g of C^{14} -Lipid II has been isolated from *Micrococcus luteus*, the amount isolated is minuscule, impure and radioactive. (see, i.e., Brotz, H., et al., *Antimicrob. Agents Chemother*, **42**, 154 (1998).) Secondly, complete separation of the miniscule amount of Lipid II from relatively large amounts of cellular lipid and membrane components is very difficult. Hence, there is a need for an ample and sustainable supply of Lipid II to support any meaningful study of the transglycosylation process.

While Lipid II is a useful tool in the study of resistance mechanisms at the molecular level and the search for newer and more effective agents, it has other uses as well. For instance, Lipid II is useful in the quantitation of lysozyme, an enzyme that preferentially hydrolyzes the beta-1,4 glucosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine that occur, for example, in the

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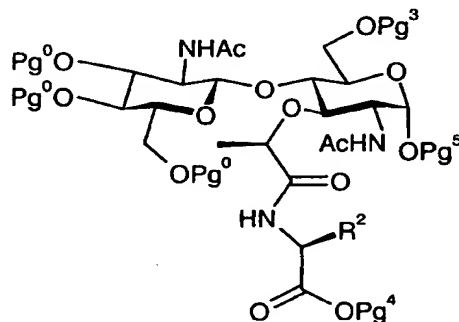
mucopeptide cell wall structure of microorganisms such as *Micrococcus lysodeikticus*. In addition, certain lantibiotics can be purified by affinity chromatography on a resin containing immobilized Lipid II.

5

BRIEF SUMMARY OF THE INVENTION

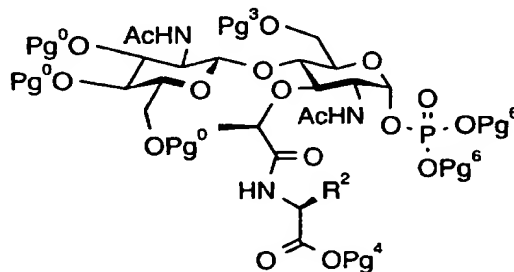
The invention provides a process for preparing a lipid substrate (e.g., Lipid II analogs) that may be used as a substrate for transglycosylase enzymes of bacterial cell wall biosynthesis. The process includes the steps of:

- (1) providing a protected disaccharide of formula 14



14

- (2) introducing an anomeric phosphate to form a compound of formula 12

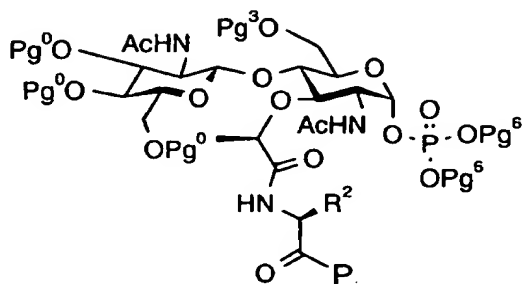


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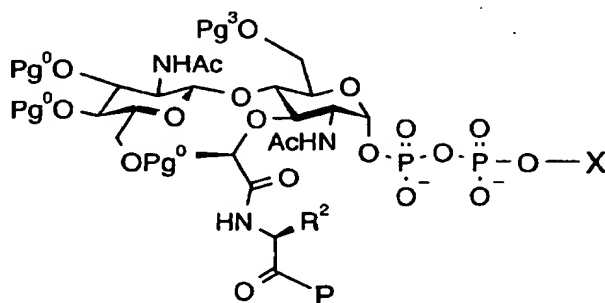
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(3) introducing a peptide linkage to form a compound of formula 7



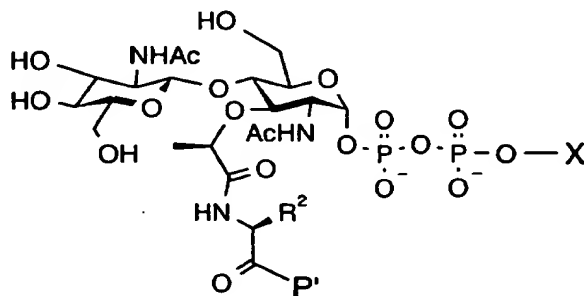
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(4) introducing a lipid-carrier diphosphate linkage to form a compound of formula 2



2

(5) removing the PG^0 and PG^3 groups and deprotecting the P group to produce a lipid substrate of formula 1



1

10 wherein:

Ac is $-C(O)CH_3$;

Pg^0 is an acyl hydroxy-protecting group;

Pg^3 is an acyl hydroxy-protecting group;

Pg^4 is a carboxy-protecting group;

5 Pg^5 is a hydroxy-protecting group;

Pg^6 is a phosphate-protecting group;

R^2 is hydrogen, (C_1-C_5) alkyl or (C_1-C_3)
alkylphenyl;

X is a lipid carrier;

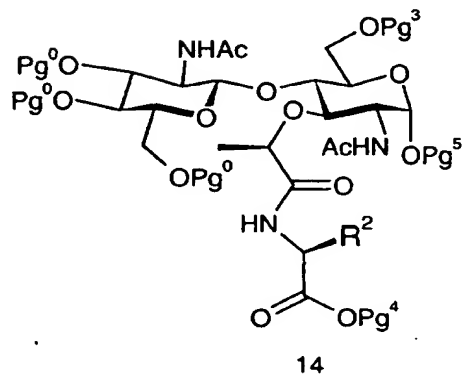
10 P attached to the carbonyl is a residue of an
amino acid or peptide, wherein P comprises a protected
terminal carboxy group; and

P' is a residue of an amino acid or peptide.

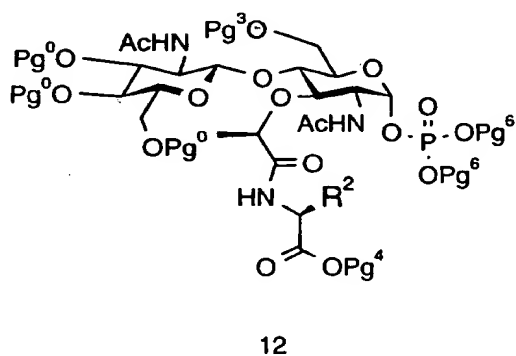
15 The invention also provides a chemical synthesis of
Lipid II which provides a non-radioactive, sustainable
supply having an isolated purity $\geq 50\%$ (preferably $\geq 60\%$,
70% or 80%, more preferably $\geq 90\%$ or 95%, even more
preferably $\geq 98\%$ or 99%, even more preferably $\geq 99.5\%$). The
20 process for the preparation of Lipid II comprises the steps
of:

(1) providing a protected disaccharide of formula 14

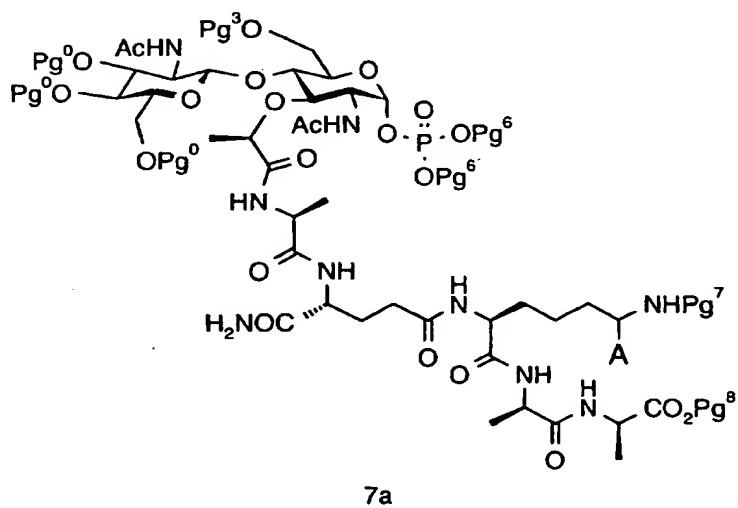
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(2) introducing an anomeric phosphate to form a compound of formula 12

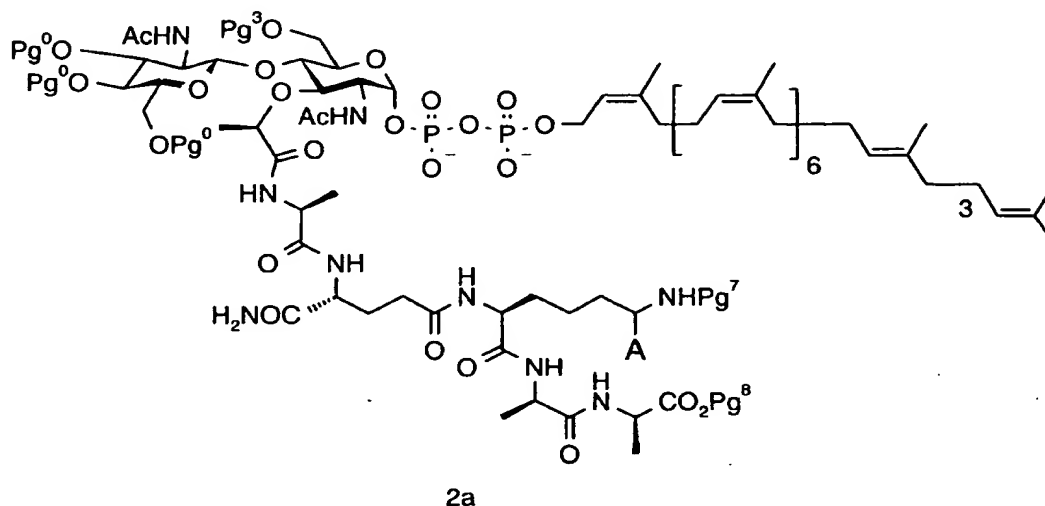


5 (3) introducing a peptide linkage to form a compound of formula 7a



-7-

(4) introducing an undecaprenyl diphosphate linkage to form a compound of formula 2a



(5) removing Pg^0 , Pg^3 , Pg^7 , and Pg^8 to form Lipid II;

5 wherein:

A is hydrogen or a carboxyl group;

R^2 is methyl;

Ac is $-C(O)CH_3$;

Pg^0 is an acyl hydroxy-protecting group;

10 Pg^3 is an acyl hydroxy-protecting group;

Pg^4 is a carboxy-protecting group;

Pg^5 is a hydroxy-protecting group;

Pg^6 is a phosphate protecting group;

Pg^7 is an amine-protecting group; and

15 Pg^8 is a carboxy-protecting group.

The invention further provides a substantially pure form of Lipid II. The Lipid II substrate is synthetically

produced using the process described above and purified by means of chromatography.

Finally, the invention provides a process for isolating Lipid II, wherein the Lipid II is isolated at a pH greater than 6 (preferably from 6 to 12, more preferably from 7 to 10, even more preferably from 7 to 9).

Detailed Description

Definitions

As used above, and throughout the description of the invention, the following abbreviations, unless otherwise indicated, shall be understood to have the following abbreviations:

	<u>Designation</u>	<u>Reagent or Fragment</u>
15	Ac	-C(O)CH ₃
	AcOH	acetic acid
	Ac ₂ O	acetic anhydride
	BOC	t-butyloxycarbonyl
	DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
20	TsOH	p-toluenesulfonic acid
	NMM	N-methylmorpholine
	THF	tetrahydrofuran
	Bn	Benzyl (i.e., -CH ₂ Ph)
	TFA	trifluoroacetic acid
25	Troc	2,2,2-trichloroethoxycarbonyl

	Cbz	benzyloxycarbonyl
	TLC	thin layer chromatography
	NMR	nuclear magnetic resonance
	ESI-MS	electro-spray ionization mass
5		spectrometry
	EtOAc	ethyl acetate
	IR	infrared spectroscopy
	MeOH	methanol
	NaOMe	sodium methoxide
10	NHS	N-hydroxysuccinimide
	EDCI	1-[3-(dimethylamino)propyl]-3- ethylcarbodiimide hydrochloride
	Ph	phenyl
	CDI	1,1'-carboxyldiimidazole
15	h	hour(s)
	min	minutes

As used above, and throughout the description of the invention, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"Amino acid" means an amino acid selected from the group consisting of natural and unnatural amino acids as defined herein. Amino acid is also meant to include α -amino acids having L or D stereochemistry at the α -carbon.

Preferred amino acids are those possessing an α -amino group.

The amino acids may be neutral, positive or negative depending on the substituents in the side chain. "Neutral amino acid" means an amino acid containing uncharged side chain substituents. Exemplary neutral amino acids include

5 alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine and cysteine. "Positive amino acid" means an amino acid in which the side chain substituents are positively charged at physiological pH. Exemplary positive

10 amino acids include lysine, arginine and histidine. "Negative amino acid" means an amino acid in which the side chain substituents bear a net negative charge at physiological pH. Exemplary negative amino acids include aspartic acid and glutamic acid. Preferred amino acids are

15 α -amino acids. Exemplary natural amino acids are isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine, histidine, aspartic acid and glutamic acid. "Unnatural amino acid" means an amino acid

20 for which there is no nucleic acid codon. Examples of unnatural amino acids include, for example, the D-isomers of the natural α -amino acids as indicated above; Aib (aminobutyric acid), β Aib (3-amino-isobutyric acid), Nva (norvaline), β -Ala, Aad (2-aminoadipic acid), β Aad (3-

25 aminoadipic acid), Abu (2-aminobutyric acid), Gaba (γ -

aminobutyric acid), Acp (6-aminocaproic acid), Dbu (2,4-diaminobutryic acid), α -aminopimelic acid, TMSA (trimethylsilyl-Ala), aIle (allo-isoleucine), Nle (norleucine), tert-Leu, Cit (citrulline), Orn, Dpm (2,2'-diaminopimelic acid), Dpr (2,3-diaminopropionic acid), α - or β -Nal, Cha (cyclohexyl-Ala), hydroxyproline, Sar (sarcosine), or the like; cyclic amino acids; N^a-alkylated amino acids such as MeGly (N^a-methylglycine), EtGly (N^a-ethylglycine) and EtAsn (N^a-ethylasparagine); and amino acids in which the α -carbon bears two side-chain substituents. The names of natural and unnatural amino acids and residues thereof used herein follow the naming conventions suggested by the IUPAC Commission on the Nomenclature of Organic Chemistry and the IUPAC-IUB Commission on Biochemical Nomenclature as set out in "Nomenclature of α -Amino Acids (Recommendations, 1974) " Biochemistry, 14(2), (1975). To the extent that the names and abbreviations of amino acids and residues thereof employed in this specification and appended claims differ from those noted, differing names and abbreviations will be made clear.

"Amino acid protecting group" and "peptide-protecting group" mean a group that protects an acid or amine moiety of the amino acid/peptide or other reactive moiety on the side chain of an amino acid/amino acid residue, e.g., hydroxy or

thiol. For examples of "corresponding protected derivatives" of amino acid side chains, see T.W. Green and P.G.M. Wuts in "Protective Groups in Organic Chemistry" John Wiley and Sons, 1991. Protecting groups for an acid group
5 in an amino acid are described herein in the section "carboxy-protecting group." Protecting groups for an amine group in an amino acid are described in the section "amine-protecting group."

"Amino acid residue" means the individual amino acid
10 units incorporated into a peptide, or peptide portion of a molecule, through an amide linkage.

"Amine-protecting group" means an easily removable group that is known in the art to protect an amino group against undesirable reaction during synthetic procedures and
15 to be selectively removable. The use of amine-protecting groups is well known in the art for protecting groups against undesirable reactions during a synthetic procedure and many such protecting groups are known, for example, T.H. Greene and P.G.M. Wuts, Protective Groups in Organic
20 Synthesis, 2nd edition, John Wiley & Sons, New York (1991), incorporated herein by reference. Amine protecting group also includes acid-labile amine-protecting groups (e.g., BOC) and hydrogenation-labile amine-protecting groups (e.g., Cbz). A preferred Pg^7 amine-protecting group is
25 trifluoroacetyl. In the present invention, Pg^2 is a group which does not lead to the generation of undesirable

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oxazoline by-products (i.e., Pg^2 cannot be an acyl group). Suitable Pg^2 amine-protecting groups include carbamate and imide groups. Particular imide groups include phthalimide, tetrachlorophthalimide and $(Ac)_2N-$. Particular carbamate groups include methoxy-carbonyl, 9-fluorenylmethoxycarbonyl, 2,2,2-trifluoroethoxycarbonyl, 2-trimethylsilylethoxycarbonyl, vinyloxycarbonyl, allyloxycarbonyl, t-butylloxycarbonyl (BOC), 1,1-dimethyl-propynyloxycarbonyl, benzyloxycarbonyl (CBZ), p-nitrobenzyloxycarbonyl, 2,4-dichloro-benzyloxycarbonyl, trimethylsilyloxycarbonyl, 2,2,2-trichloroethoxycarbonyl, 1,1-dimethyl-2,2,2-trichloroethoxycarbonyl, or the like. A preferred Pg^2 amine-protecting group is 2,2,2-trichloroethoxycarbonyl.

"Carboxy-protecting group" means an easily removable group that is known in the art to protect an acidic hydrogen of a carboxyl group against undesirable reaction during synthetic procedures, e.g., to block or protect the acid functionality while the reactions involving other functional sites of the compound are carried out, and to be selectively removable. Such acid protecting groups are well known to those skilled in the art, having been extensively used in the protection of carboxyl groups, as described in U.S. Pat. No. 3,840,556 and 3,719,667, the disclosures of which are hereby incorporated herein by reference. For suitable acid protecting groups, see T.W. Green and P.G.M. Wuts in "Protective Groups in Organic Chemistry" John Wiley and

Sons, 1991. Acid protecting group also includes hydrogenation labile acid protecting groups, such as benzyl. Examples of acid protecting groups include esters such as substituted and unsubstituted C₁ to C₈ alkyl, e.g., methyl, 5 ethyl, t-butyl, methoxymethyl, methylthiomethyl, 2,2,2-trichloroethyl or the like, tetrahydropyranyl, substituted and unsubstituted phenylalkyl such as benzyl and substituted derivatives thereof such as alkoxybenzyl or nitrobenzyl groups or the like, cinnamyl, dialkylaminoalkyl, e.g., 10 dimethylaminoethyl or the like, trimethylsilyl, substituted and unsubstituted amides and hydrazides, e.g., amides and hydrazides of N,N-dimethylamine, 7-nitroindole, hydrazine, N-phenylhydrazine or the like, acyloxyalkyl groups such as pivaloyloxymethyl or propionyloxymethyl or the like, 15 aroyloxymethyl such as benzoyloxymethyl or the like, alkoxy-carbonylalkyl such as methoxycarbonylmethyl, cyclohexyloxycarbonylmethyl or the like, alkoxy-carbonyloxyalkyl such as t-butyloxycarbonyloxymethyl or the like, alkoxy-carbonylaminoalkyl such as t- 20 butyloxycarbonylaminomethyl or the like, alkylaminocarbonylaminoalkyl, such as methylaminocarbonylaminomethyl or the like, acylaminoalkyl such as acetylaminomethyl or the like, heterocyclylcarbonyloxyalkyl such as 4-methylpiperazinyl- 25 carbonyloxymethyl or the like, dialkylaminocarbonylalkyl such as dimethylaminocarbonyl-methyl or the like, (5-(lower

alkyl)-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-*t*-butyl-2-oxo-1,3-dioxolen-4-yl)methyl or the like, and (5-phenyl-2-oxo-1,3-dioxolen-4-yl)alkyl such as (5-phenyl-2-oxo-1,3-dioxolen-4-yl)methyl or the like. Particular carboxy-protecting groups include methyl, 9-fluorenylmethyl, 2-(trimethylsilyl)ethyl, 2-(trimethylsilyl)ethoxymethyl, 2-methylthioethyl, 1,3-dithianyl-2-methyl, 2-(*p*-toluenesulfonyl)ethyl, 2-(*p*-nitrophenylsulfonyl)ethyl, 2-(2'-pyridyl)ethyl, 2-(diphenylphosphino)ethyl, *p*-(methylmercapto)phenyl, nitroethyl, allyl or the like. A preferred Pg⁴ carboxy-protecting is -CH₂CH₂SO₂Ph. A preferred Pg⁸ carboxy-protecting group is methyl.

"Hydroxy-protecting group" means an easily removable group that is known in the art to protect an hydroxyl group against undesirable reaction during synthetic procedures and to be selectively removable. The use of hydroxy-protecting groups is well known in the art for protecting groups against undesirable reactions during a synthetic procedure and many such protecting groups are known, for example, T.H. Greene and P.G.M. Wuts, Protective Groups in Organic Synthesis, 2nd edition, John Wiley & Sons, New York (1991), incorporated herein by reference. In the present invention, the Pg⁰, Pg¹, and Pg⁵ hydroxy-protecting groups are mutually orthogonal, as described herein. Pg¹ cannot be an electron-withdrawing group, since such groups deactivate the coupling reaction between the muramylamide compound of formula 18 and

glucosopyranosyl compound of formula 17. Suitable Pg^1 groups include aralkyl, aralkenyl and silyl groups. Particular aralkyl and alkenyl groups include benzyl and allyl, respectively. Particular silyl groups include

5 trialkylsilyl groups, such as trimethylsilyl and (t-butyl)dimethylsilyl. Preferred Pg^1 groups are allyl and benzyl; a more preferred group is benzyl. Suitable Pg^5 groups include aralkyl and alkenyl. Preferred Pg^5 groups include allyl, n-pentenyl, and benzyl; a more preferred

10 group is benzyl. In addition, Pg^0 and Pg^3 must be removable by saponification (i.e. Pg^0 and Pg^3 must be acyl groups). Particular acyl groups include formyl, acetyl, chloroacetyl, trichloroacetyl, o-nitrophenylacetyl, o-nitrophenoxy-acetyl, trifluoroacetyl, acetoacetyl, 4-chlorobutyryl, isobutyryl,

15 o-nitrocinnamoyl, picolinoyl, acylisothiocyanate, aminocaproyl, benzoyl, or the like. Preferred Pg^0 and Pg^3 groups are chloroacetyl and acetyl; a more preferred group is acetyl.

"Phosphate-protecting group" means an easily removable

20 group that is known in the art to protect a phosphate group against undesirable reaction during synthetic procedures and to be selectively removable. The use of phosphate-protecting groups is well known in the art for protecting groups against undesirable reactions during a synthetic

25 procedure and many such protecting groups are known, for example, T.H. Greene and P.G.M. Wuts, Protective Groups in

Organic Synthesis, 2nd edition, John Wiley & Sons, New York (1991), incorporated herein by reference. A preferred Pg⁶ phosphate-protecting group is benzyl.

"Leaving group" of an activated ester means a
5 substituent having sufficient lability such that it can be substituted by a good nucleophile (e.g., an amino group of a peptide unit). The lability of a particular substituent will vary depending upon substituents on the same and/or adjacent carbon atoms and the nature of the leaving group.
10 Those skilled in the art will appreciate the types of leaving groups capable of substitution by an amino nucleophile. For suitable leaving groups, see M. Bodanszky and A. Bodanszky in "The Practice of Peptide Synthesis" Springer-Verlag, 1984; and M. Bodanszky in "Princliples of
15 Peptide Synthesis" Springer-Verlag, 1984. In the present invention, for example, the leaving group activates the attached carbonyl such that the terminal amino acid group acts as a linker for linking the disaccharide with the peptide unit. Particular leaving groups include
20 pentafluorophenoxy, N-oxysuccimide, N-oxypthalimide, and N-oxymethyltriazole. A preferred leaving group is N-oxysuccinimide.

"Orthogonal protecting groups" means protecting groups for which there exists a set of conditions wherein one of
25 the groups can be removed without removing the other(s). The term encompasses protecting groups for different moieties

(e.g., orthogonal amine and hydroxy protecting groups) as well as the same moiety (e.g., orthogonal hydroxy-protecting groups). It is not a requirement that orthogonal protecting groups necessarily be different. For example, when the term
5 is used to describe protecting groups for the same moiety, the groups may be different (e.g., orthogonal acetyl and benzyl hydroxy-protecting groups) or the same (e.g., orthogonal benzyl protecting groups).

"Electron-withdrawing group" means a group which is a
10 more powerful electron attractor than hydrogen. Electron withdrawing groups exhibit negative inductive effects, whereas groups which are poorer electron attractors than hydrogen exhibit positive inductive effects. (see, e.g., E. S. Gould, Mechanism and Structure in Organic Chemistry,
15 Holt, Rinehart and Winston, New York (1959), incorporated herein by reference).

"Acyl" means an $R-C(O)-$ group, wherein R is bonded to the CO group through a carbon-carbon bond.

"Alkyl" means an aliphatic hydrocarbon group which may
20 be straight or branched having about 1 to about 20 carbon atoms in the chain. Preferred alkyl groups have 1 to about 12 carbon atoms in the chain, more preferred is lower alkyl as defined herein. Branched means that one or more lower alkyl groups such as methyl, ethyl or propyl are attached to
25 a linear alkyl chain. "Lower alkyl" means about 1 to about

4 carbon atoms in the chain that may be straight or branched.

"Alkenyl" means an aliphatic hydrocarbon group containing a carbon-carbon double bond and which may be straight or branched having about 2 to about 15 carbon atoms in the chain. Preferred alkenyl groups have 2 to about 12 carbon atoms in the chain; and more preferably about 2 to about 4 carbon atoms in the chain. Branched means that one or more lower alkyl groups such as methyl, ethyl or propyl are attached to a linear alkenyl chain. "Lower alkenyl" means about 2 to about 4 carbon atoms in the chain that may be straight or branched. Exemplary alkenyl groups include ethenyl, propenyl, *n*-butenyl, *i*-butenyl, 3-methylbut-2-enyl, *n*-pentenyl, heptenyl, octenyl, cyclohexylbutenyl and decenyl.

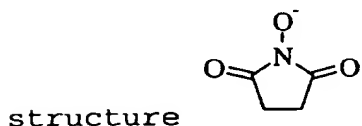
"Aryl" means an aromatic monocyclic or multicyclic ring system of about 6 to about 14 carbon atoms, preferably of about 6 to about 10 carbon atoms. Exemplary aryl groups include phenyl or naphthyl, or phenyl substituted or naphthyl substituted.

"Base of formula B" and "base of formula B²" mean a compound comprising a SP² and SP³ hybridized nitrogen having a non-bonded pair of electrons which is capable of being protonated. Examples of Bases of formula B or B¹ include compounds comprising optionally substituted imino,

optionally substituted amino, and optionally substituted amidino groups.

"Carboxy" means an $\text{HO}(\text{O})\text{C}-$ (carboxylic acid or salt thereof) group.

5 "N-oxy succinimide" means a moiety of the following



"Lipid carrier" means saturated and unsaturated hydrocarbon chains having more than one carbon. The chains may be straight or branched. The hydrocarbon may also be substituted (e.g., perfluorinated) or unsubstituted.

10

Preferably, the hydrocarbon chain contains from 5 to 55 carbons and 1 to 11 prenyl units, more preferably 25 to 55 carbons and 5 to 11 prenyl units, most preferably 40 to 55 carbons and 8 to 11 prenyl units.

15 "Peptide" means a polymer encompassing amino acid residues joined together through amide bonds. Suitable peptide and polypeptide units include peptides containing 2 or more, preferably 2 to 4, amino acid residues. P can also be a single amino acid residue. P is preferably a

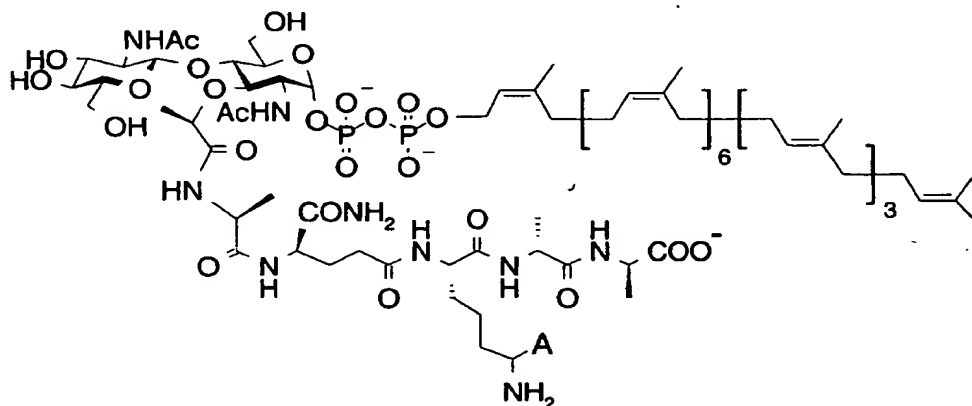
20 polypeptide containing 4 amino acid residues. Particularly useful polypeptide units are D-iGln-L-Lys-D-Ala-D-Ala or D-iGln-Dap-D-Ala-D-Ala. Other combinations of amino acids and number of amino acids included in the amino acid or peptide unit may be used depending upon the enzyme selected to

interact with the Lipid substrate. Suitable amino acid residues include natural amino acids, unusual amino acids and modified amino acids as defined in the World

Intellectual Property Organization (WIPO) Handbook on

- 5 Industrial Property Information and Documentation, Standard ST.25: Standard for the Presentation of Nucleotide and Amino Acid Sequence Listings in Patent Applications (1998), including Tables 1 through 6 in Appendix 2, incorporated herein by reference.

- 10 "Lipid II" means a compound of the following structure:



- where A is a hydrogen or a carboxyl group ($-\text{CO}_2\text{H}$ or salt thereof); Ac is $-\text{C}(\text{O})\text{CH}_3$ and W^+ is each independently a proton or cation selected from the group consisting of an alkali metal (e.g., sodium or potassium), alkaline earth metal (e.g., magnesium or calcium), ammonium, alkyl ammonium (e.g., methyl ammonium or ethylammonium), and dialkyl ammonium (e.g., dimethyl ammonium, methylethylammonium or diethylammonium). Preferably, the pyrophosphate group is

not protonated. For gram-positive Lipid II, A is generally hydrogen; whereas, for gram-negative Lipid II, A is generally a carboxyl group.

"Substantially pure" means an isolated purity of
5 greater than or equal to 99% purity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the transglycosylation process using Lipid II as a substrate in cartoon form.

10 Figure 2 illustrates the fluorescence detection of glycan strands generated by incubating a synthesized dansyl-tagged Lipid II derivative with a monofunctional transglycosylase (MtgA) from *Staphylococcus aureus*.

15 ***Utility of Lipid II and Analogs Thereof***

I. Quantitation of Lysozyme Using Lipid II

Lipid II is useful in the quantitation of lysozyme, an enzyme that preferentially hydrolyzes the beta-1,4 glucosidic linkages between N-acetylmuramic acid and N-
20 acetylglucosamine that occur, for example, in the mucopeptide cell wall structure of microorganisms such as *Micrococcus lysodeikticus*.

Lysozyme may be the mediator in the anti-tumor function of macrophages (Osserman et al. (1973) *Nature* 243:331)
25 which, it has been shown, secrete this enzyme

(Gordon et al. (1974) *J. Exp. Med.* 139:1228). Cartilage lysozyme may play a role in cartilage calcification (Kuettnner et al. (1974) *Biochim. Biophys. Acta* 372:335). There has been interest in lysozyme as a "natural"

5 antibiotic, and as an aid in the diagnosis of disease (Glynn (1968) *Sci. Basis Med. Ann. Rev.* 31; Pruzanski et al. (1969) *Amer. J. Med. Sci.* 258:405). Elevated levels of serum and urinary lysozyme are present in monocytic and mono-
myelocytic leukemia (Osserman et al. (1966) *J. Exp. Med.*
10 124:921; Brierre et al. (1974) *Clin. Chim. Acta* 50:265). The presence of this enzyme in cerebrospinal fluid is indicative of tumors of the central nervous system (Newman et al. (1974) *Lancet II* 756). Normally, lysozyme activity is practically absent from urine, bile, and spinal fluid
15 (Hankiewicz et al. (1974) *Clin. Chim. Acta* 57:205). The enzyme is also used for lysing *E. coli* and *Streptomyces* for extraction purposes (Haas et al. (1975) *Methods in Enzymology*, XLIII (Hash, J., ed.), Academic Press, N.Y., p. 621), such as extracting group specific antigen (Watson et
20 al. (1975) *J. Clin. Microbiol.* 1:274).

Thus, lysozyme has a number of important medical and research implications and uses, and accurate quantitation thereof is therefore important in these applications.

Initially, lysozyme activity was estimated via a cell
25 turbidimetric assay using *Micrococcus luteus* as a substrate.

-24-

The rate of lysis of the cells by lysozyme is determined spectrophotometrically by measuring the decrease in turbidity of the cell solution. However, this method is not reliably reproducible due to lack of uniformity of *M. luteus* cell powder. The results also vary widely with buffer ionic strength differences.

Presently, a commercially available substrate for colorimetric assessment of lysozyme activity can be purchased from Sigma Chemical Company (St. Louis, MO). The substrate is the synthetic trimeric sugar, *p*-nitrophenyl beta-D-*N,N',N''*-triacetylchitotriose (PNP-(GlcNAc)₃) (Sigma Catalog product no. N-8638). Longer and shorter acetylchitooligo-saccharides have also been utilized for lysozyme assays, i.e., PNP-(GlcNAc)_n, where *n* = 2-5. In all cases, the favored cleavage site for lysozyme results in the formation of PNP-GlcNAc. The activity measured in this assay is the increase in absorbance at 405 nm of the liberated *p*-nitrophenol. However, cleavage of the substrate by lysozyme to PNP-GlcNAc does not directly result in the liberation of *p*-nitrophenol. To obtain reliable colorimetric measurements of lysozyme activity, one must couple the assay to a second enzyme, β -*N*-acetylhexosaminidase (NAHase). NAHase cleaves PNP-GlcNAc to liberate *p*-nitrophenol, which is then quantitated (Nanjo et al. (1988) *J. Biochem.* 104:255-258). This assay, while more

accurate than earlier assays, still results in a high level of error due to lysozyme cleavage of the substrate that produces cleavage products other than PNP-GlcNAc. Also, two enzyme systems are less preferential than a system that
5 directly measures the activity of interest. A new and improved assay that is highly accurate and easy to use would be valuable in laboratory settings for the routine determination of lysozyme in pharmaceutical/diagnostic preparations and biological materials. Such an assay can
10 be utilized as an accurate diagnostic tool for the diagnosis of leukemia and tumors of the central nervous system. Detection of lysozyme activity in human serum, urine, or cerebrospinal fluid would quickly and accurately assist in the diagnosis of certain cancers, as noted above, and would
15 be a valuable addition to the arsenal of cancer diagnostic tools. Lipid II can be used in lysozyme assays as follows.

Lipid II labeled with Oregon Green at the ϵ -amine of the lysine amino acid of the pentapeptide according to the manufacturer's instructions (Molecular Probes, Eugene, OR)
20 is a fluorescent molecule. This labeled Lipid II derivative can be polymerized using *Staphylococcus aureus* monofunctional transglycosylase A (MtgA; U.S. Patent Nos. 6,143,868 and 5,922,540) into peptidoglycan strands that are non-fluorescent. Polymerization is achieved using 100 μ M
25 Oregon Green-labeled Lipid II, 5 μ M MtgA, 50 mM MES (pH

5.9), 25 mM MgCl_2 , 16.7 mM NaCl , and 0.27 mM CHAPS.

Polymerization is complete after approximately 5 hours at 25°C.

The adjacent fluorophores (either neighboring dyes or those four repeating disaccharide units away) of the polymer interact when in close proximity, resulting in self-quenching. Addition of lysozyme to the labeled peptidoglycan material results in cleavage of the same bonds formed by MtgA in the polymerization reaction. Upon cleavage of these bonds, the dyes become physically separated, resulting in a loss of interaction between the dye molecules and a subsequent rise in fluorescence due to the decrease in self-quenching. Following the fluorescence of the lysozyme-catalyzed reaction (Excitation: 495nm; Emission: 521nm) over time compared to that of a standard curve permits one to quantitate the activity of the lysozyme present in the target preparation.

Thus, a lysozyme assay kit can contain pre-polymerized chains of Oregon Green labeled peptidoglycan, for which Lipid II is a mandatory starting material, size fractionated into lengths that immediately result in an increase in fluorescence upon initial lysozyme cleavage. For example, when cleaved, dimers are certain to result in an increase in fluorescence. Alternatively, peptidoglycan polymers can be labeled with Oregon Green such that the label moieties are spaced close enough to result in complete quenching, but

sufficiently far apart that initial cleavage results in immediate relief of quenching. The amount of Oregon Green label incorporated into the polymer can be controlled by varying the ratio of labeled and non-labeled Lipid II used in the polymerization reaction. The labeled peptidoglycan can be present in the kit either as an aqueous solution or a solid powder. An aqueous solution of such Oregon Green labeled peptidoglycan can be prepared in a buffer of choice, and aliquots of the lysozyme preparation to be quantitated can then be added. Monitoring the increase in fluorescence intensity over time (Excitation 495nm; Emission: 521nm) and comparing the results to those obtained from a standard curve provides a quantitative measure of the lysozyme activity in the enzyme preparation.

15

II. Purification of Lantibiotics Using Lipid II

Lantibiotics are a class of peptide antibiotics characterized by specific post-translational modifications that result in the formation of the rare thioether amino acids lanthionin and/or 3-methyl-lanthionin within these molecules, hence the name "lantibiotics" (lanthionin-containing antibiotics). The lantibiotics epidermin, gardimycin (actagardine), mersacidin, and nisin possess antibacterial activity against gram-positive organisms.

20

Commercially, Nisin Z has been used as a food preservative for several decades, particularly in the dairy industry. It is nontoxic to humans, and the development of resistance to this antibiotic has not been observed despite
5 its prolonged use. Nisin Z is presently being considered as both a drug candidate for human use and for inclusion in medical devices and surfaces of food processing machines to prevent microbial growth. Epidermin, gardimycin, and mersacidin are potentially useful as antibacterial drugs for
10 the treatment of bacterial infections in humans.

Epidermin, mersacidin, and nisin specifically interact with Lipid II (Broetz et al. (2000) *Journal of Antimicrobial Chemotherapy* 46:1-6; Breukink et al. (1999) *Science* 286:2361-2364; Broetz et al. (1998) *Antimicrobial Agents and*
15 *Chemotherapy* 42(1):154-160). While there is no direct evidence for the binding of gardimycin to Lipid II, such binding is expected to occur in view of the structural similarities between gardimycin and mersacidin. One would therefore fully expect that all these lantibiotics can be
20 purified from a mixture of compounds, such as a culture supernatant from a lantibiotic-producing microbial strain, by affinity chromatography on a resin containing immobilized Lipid II. Examples of lantibiotic-producing strains include *Lactococcus lactis* ssp. *lactis* (nisin); *Staphylococcus*
25 *epidermidis* (epidermin); *Bacillus* sp. Strain HIL Y-85,54728 (mersacidin); and *Actinoplanes liguriae* (gardimycin).

For this purpose, Lipid II is coupled to activated resins, for example those commercially available from suppliers such as Amersham Pharmacia Biotech. Coupling is performed according to the manufacturer's instructions. Generally, a solution of the desired ligand, in this case Lipid II, is simply mixed with a suspension of the activated resin in a suitable solvent or buffer (determined by the solubility of the ligand and by the coupling chemistry used), incubated, and excess Lipid II ligand is washed away.

10 If this method of coupling results in an orientation of Lipid II relative to the surface of the resin that does not allow binding of lantibiotics, then Lipid II is bound to hydrophobic beads to overcome this problem. In this way, Lipid II is presented the same way it is found in its
15 natural membrane environment. A monolayer of Lipid II is formed on hydrophobic beads as described for a phospholipid monolayer by Retzinger et al. ((1985) *Analytical Biochemistry* 150:131-140) or Kim et al. ((1997) *Analytical Biochemistry* 250(1):109-116). For example, polystyrene-
20 divinylbenzene beads are sonicated together with Lipid II in hexane/ethanol (approx. 1/1, v/v), and dried. The beads are redispersed in water, and washed with water, resulting in a lipid monolayer on the surface of the hydrophobic beads.
If the molecular shape of Lipid II is incompatible with the
25 formation of a Lipid II-only monolayer on the surface of the

beads, the beads are coated with a mixture of phospholipids and Lipid II.

The lantibiotic-containing mixture is then passed through the column containing Lipid II resin under
5 conditions that allow binding of the lantibiotics to the immobilized Lipid II. For example, conditions shown (Broetz et al. (1998) *Antimicrobial Agents and Chemotherapy* 42(1):154-160) to allow the binding of mersacidin, and presumably gardimycin, to Lipid II include:

- 10 a) 50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 23°C; or
 b) half-concentrated Mueller-Hinton broth.

Conditions under which epidermin and nisin bind to Lipid II (Broetz et al. (1998) *Molecular Microbiology* 30(2):317-327) include:

- 15 a) 69 mM Tris-HCl, pH 8.8, 58 mM MgCl₂, 11.6 mM NH₄Cl, 5.8 mM SDS; or
 b) 10 mM Tris-HCl, pH 7.2, 0.85% NaCl.

Additional conditions under which nisin binds to Lipid II (Wiedemann et al. (2001) *Journal of Biological Chemistry*
20 276(1):1772-1779) include 25 mM MES-KOH, pH 6.0, 50 mM K₂SO₄, or 50 mM MES-KOH, pH 6.0, 100 mM K₂SO₄.

Unbound, non-lantibiotic materials are removed by eluting the column with the same buffer used for lantibiotic binding to resin-immobilized Lipid II.

It is fully expected that mersacidin and gardimycin can be eluted from the column by washing the resin with a buffer, e.g., 50 mM Tris-HCl, pH 7.8, containing EDTA, to remove divalent cations. Alternatively, bound Lipid II can
5 be acid-hydrolyzed in order to break the bond between MurNAC and phosphate, generating fragments that no longer interact with mersacidin or gardimycin.

It is also fully expected that the interactions of nisin and/or epidermin with Lipid II covalently bound to the
10 resin are broken with an organic solvent e.g., 100% acetonitrile, or a detergent. Extreme pH, high salt concentrations, or aqueous buffers containing EDTA can also be employed.

15 **Overview of Synthetic Approach**

The diversity of structural elements resident in Lipid II present several significant challenges for total synthesis. The central core of Lipid II consists of a β -[1,4]-linked disaccharide containing N-acetylglucosamine
20 (NAG) and N-acetylmuramic acid (NAM) subunits. The muramyl residue is further decorated with a pentapeptide chain (e.g., L-Ala-D-iGln-L-Lys-D-Ala-D-Ala or L-Ala-D-iGln-meso-DAP-D-Ala-D-Ala) attached to the 3-position via a lactyl linkage, as well as a chemically sensitive undecaprenyl-
25 linked α -glycosyl diphosphate moiety.

The synthesis of the central disaccharide core fragment itself in orthogonally protected form presented a significant synthetic challenge. For example, identification of a protective scheme having triple
5 orthogonality is highly desirable to accomplish selective unmasking of the three types of pendant hydroxyl groups (i.e., anomeric OH, peripheral OH, and carboxyl OH). In addition, the stereoselective construction of the β -[1,4] glycosidic linkage was expected to be difficult irrespective
10 of the method used to generate a reactive glycosyl cation donor. For example, with respect to the glycosyl cation, each of the following inherent properties contribute to a loss of reactivity of the glycosyl cation acceptor; (i) the intrinsic lack of nucleophilicity of the C(4)-hydroxyl group
15 of glucopyranose-based acceptors, (ii) additional steric crowding around the C(4)-hydroxyl of muramic acid-based acceptors, and (iii) additional electronic deactivation of 2-deoxy-2-acylaminoglucopyranose acceptors relative to their glucopyranose-based counterparts. With respect to the
20 glycosyl cation donor, an activation method with a predisposition toward formation of a β -[1,4] glycosidic linkage was needed. The reaction conditions for glycosyl cation generation also need to be compatible with functionality resident in both the donor and acceptor. Each
25 of the synthetic issues associated with stereoselective

construction of a NAG-NAM disaccharide precursor (see Scheme II below) have been addressed and a protocol for its synthesis on a multiple gram scale in fully differentiated form was developed. The synthetic process for the
5 preparation of the NAG-NAM disaccharide precursor is exemplified in the Examples. The disaccharide core may alternatively be prepared using the general procedures described in Kantoci, D., et al., *Carbohydrate Research*, **162**, 227 (1987).

10 In addition to the synthesis of the disaccharide core, Applicants have also successfully addressed other challenges in the total synthesis of Lipid II which include: (1) a method for introduction of an anomeric phosphate onto a suitably protected disaccharide precursor in an α -selective
15 manner, (2) a mild method for introduction of the chemically sensitive undecaprenyl-linked diphosphate, (3) orthogonality of protective groups employed in the pentapeptide side chain and the carbohydrate periphery with those utilized to temporarily mask reactive functionality at
20 the anomeric center of the muramyl fragment, (4) coordination of the entire protective group scheme to allow global deprotection under mild reaction conditions as the final step in the synthesis, and (5) development of a high performance liquid chromatography (HPLC) protocol for
25 purification of the final product. The synthesis of Lipid

II described below successfully addresses each of the issues outlined above.

Introduction of the undecaprenyl-linked diphosphate moiety at the later stage in the synthesis provided distinct
5 advantages. For example, it avoided potential solubility complications in subsequent reactions that could arise from the enhanced lipophilic character of the undecaprenyl-linked substrate. In addition, late stage introduction of the
lipid-linked diphosphate also minimized the number of
10 subsequent synthetic operations that the chemically sensitive allylic diphosphate linkage had to withstand.

Applicants also discovered that the use of base-cleavable protective groups for all functional groups that are unmasked subsequent to their incorporation allowed them
15 to avoid any potential acid sensitivity of the anomeric diphosphate.

Decomposition of the Lipid II intermediate due to the acid sensitivity of the pyrophosphate group has not been fully recognized in the art. Applicants discovered that
20 once the pyrophosphate moiety was present in the compound by-products from decomposition could be minimized by maintaining a pH greater than about 6. Preferably the pH is maintained from 6 to 12, more preferably from 7 to 10, most preferably from 7 to 9. It is believed that even the
25 natural product could be isolated in higher yields and purity if the pH was maintained at a pH greater than 6

-35-

instead of the current practice of using 6M pyridinium/acetate in the isolation procedure which has a pH of 4.2. For a detailed description of the current isolation procedures for the natural product, see: Van Heuenoort, Y.,
5 et al., "Membrane Intermediates in the Peptidoglycan Metabolism of *Escherichia coli*: Possible Roles of PBP 1b and PBP 3," J. Bacteriol., **174**(11), 3549-3557 (1992); and Anderson, J.S., et al., "Biosynthesis of the Peptidoglycan of Bacterial Cell Walls," J. Biol. Chem., **242**(13), 3180-3190
10 (1967)).

The Lipid II may be purified using conventional chromatography procedures well-known to those skilled in the art. A particular useful set of chromatography conditions is illustrated in the examples. The Lipid II is isolated in
15 purities much higher than currently available. One may isolate the Lipid II in purities greater than 50%. Generally, the Lipid II may be isolated in greater than or equal to 60%, 70%, 80%, 90%, 95%, or 98% purity and under optimum conditions in substantially pure form ($\geq 99\%$).

20 It is to be understood that this invention covers all appropriate combinations of the particular and preferred groupings, and individual or combination of process steps as referred to herein.

25

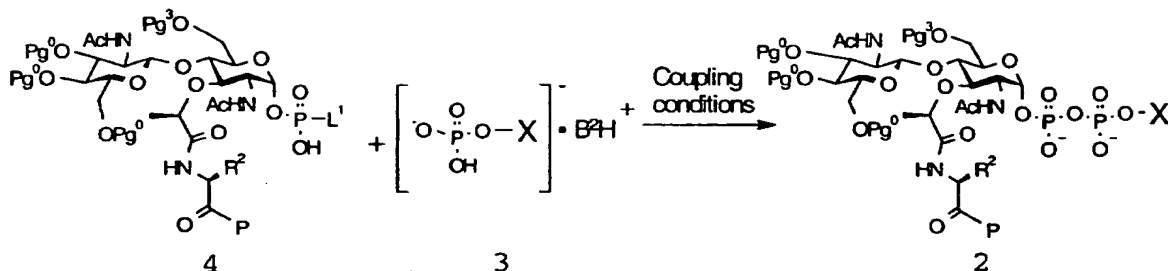
General Preparation of Lipid II Analogs

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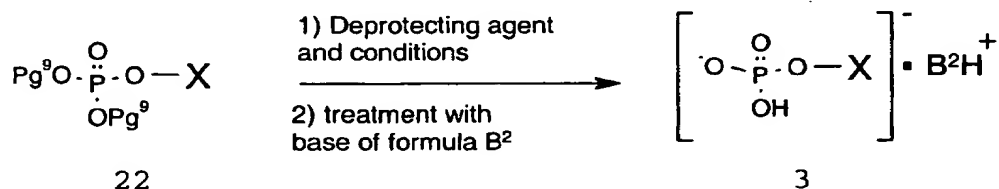
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A particular L¹ is imidazolyl, or the like. Particular conditions encompass exposing the activated phosphate to the
 5 monophosphate (Et₃NH⁺ salt), or the like in the presence of 1H-tetrazole, or the like (1 equivalent) over about 4 days in DMF/THF, or the like under about an argon atmosphere, or the like.

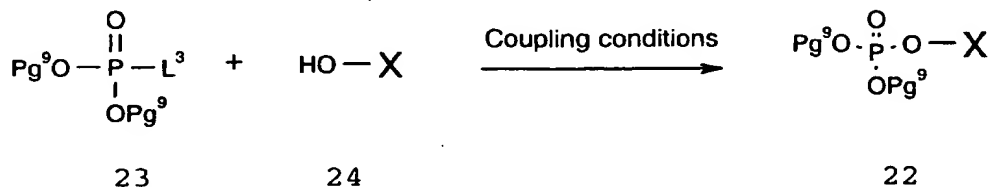
A compound of formula 3, wherein the variables are as
 10 described herein, may be prepared by (1) deprotecting a compound of formula 22, wherein Pg⁹ is a phosphate protecting group and X is as described herein, in the presence of a deprotecting agent under appropriate conditions, and (2) treating the resulting compound with a
 15 base of formula B², wherein B² is as described herein.



20 A particular Pg⁹ phosphate-protecting group is 2,2,2-trichloroethyl, or the like. A particular base of formula B² is triethylamine, or the like. A particular deprotecting agent is Zn dust, or the like, in the presence of a proton

source (e.g., HCl), or the like. Particular conditions encompass adding 0.1 M HCl, or the like in THF (2-5 mL), or the like, dropwise in two portions over about an hour to a stirred solution or suspension in THF, or the like, of the compound of formula 22 (about 1 equiv) and zinc dust (about 12 equiv), or the like, at about 0 °C. The reaction mixture is stirred at about 0 °C and followed to completion by TLC. Upon completion, the reaction mixture is partitioned between Et₂O, or the like and 1N HCl, or the like, and stirred until the zinc dust, or the like, is completely dissolved. The phases are separated and the aqueous phase extracted with Et₂O (x3), or the like. The combined Et₂O layers are dried with Na₂SO₄ and filtered. Triethylamine, or the like, (1-3 mL), is added and the organic phase concentrated in vacuo to yield compound 3 (80 - 95%).

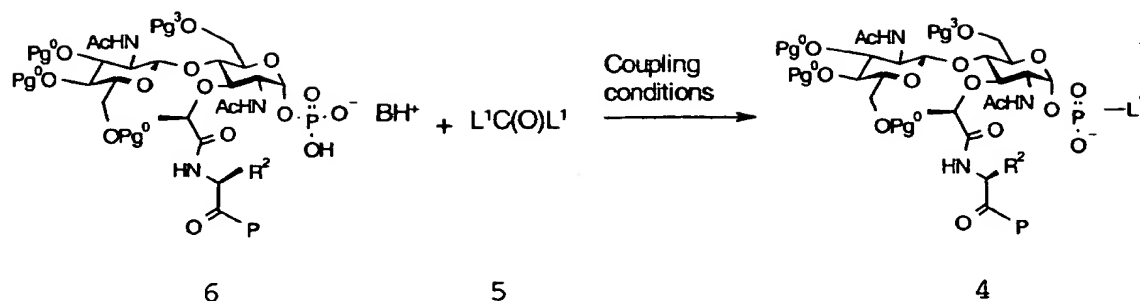
A compound of formula 22, wherein the variables are as described herein, may be prepared by coupling a compound of formula 23, wherein L³ is a leaving group and Pg⁹ is as described herein, with a lipid alcohol of formula 24, wherein X is as described herein, under appropriate conditions.



A particular $-L^3$ group is Cl, or the like. A particular Pg^9 group is 2,2,2-trichloroethyl, or the like. Particular conditions encompass adding the compound of formula 23 (about 1.5 equiv) and N,N-dimethylamino-pyridine (about 0.2
5 equiv), or the like, to a solution of the lipid alcohol (about 1 equiv) in anhydrous CH_2Cl_2 (about 0.1 M), or the like. Triethylamine (3 equiv), or the like, is added dropwise and the reaction stirred at room temperature. After completion of the reaction as evidenced by TLC, the
10 reaction mixture is partitioned between CH_2Cl_2 , or the like and 1 N HCl, or the like. The aqueous layer is extracted with CH_2Cl_2 (x3), or the like. The organic layer is washed sequentially with H_2O and brine, or the like, dried (Na_2SO_4), or the like and concentrated in vacuo. The residue is
15 purified by flash chromatography over silica gel eluting with a gradient of hexanes to about 10% EtOAc in hexanes. Concentration of relevant fractions affords the compound of formula 22 (about 80 - about 90%) as a colorless oil.

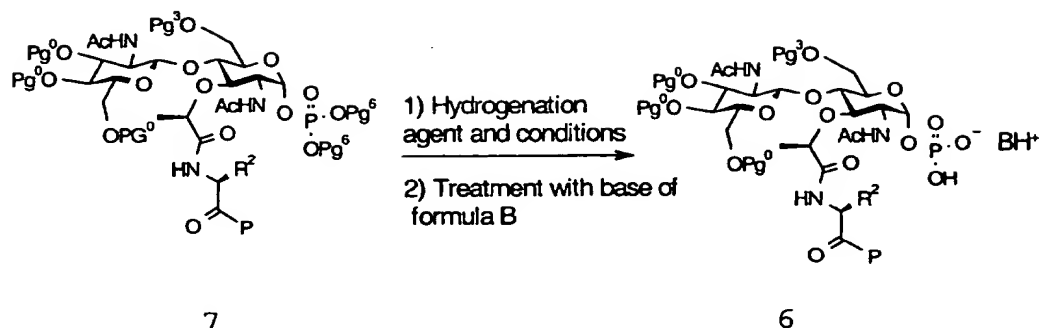
A compound of formula 4, wherein the variables are as
20 described herein, may be prepared by coupling a compound of formula 6, wherein B is a base and the other variables are as described herein, with an activating agent of formula 5 under appropriate conditions.

-40-



The coupling reaction initially yields a phosphate diester intermediate, which gives off CO_2 to yield the activated phosphate of formula 4. A particular activating agent is CDI, or the like. A particular base counter ion BH^+ is $\text{C}_5\text{H}_5\text{NH}^+$, or the like. Particular conditions encompass carrying out the reaction in anhydrous DMF/THF, or the like for about 2 h. Electrophilic activation of the anomeric phosphate by conversion to the corresponding phosphorimidazolate derivative is described in Fang, X., et al., *Bioorg. Med. Chem. Lett.*, **5**, 2701 (1995).

A compound of formula 6, wherein the variables are as described herein, may be prepared by (1) removing the Pg^6 groups of a compound of compound of formula 7, wherein the variables are as described herein, in the presence of a hydrogenation agent under appropriate conditions, and (2) treating the resulting compound with a base of formula B to form a phosphate salt.



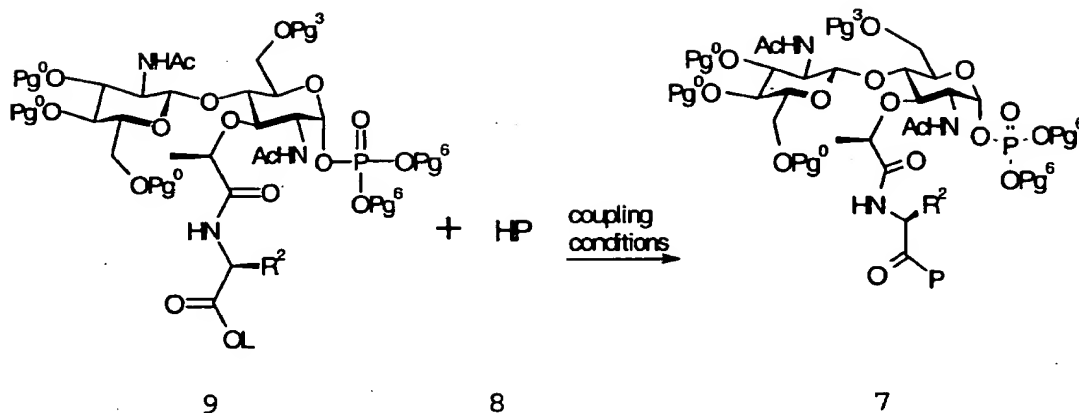
A particular Pg^6 group is benzyl, or the like. A particular hydrogenation agent is Pd/C, or the like. Particular

5 hydrogenation conditions encompass adding the compound 7 to a suspension of about 10% Pd/C, or the like in an alcohol (preferably methanol), or the like cooled in an ice bath to aid in degassing the reaction solution. The solution is then warmed to room temperature and hydrogenated at

10 atmospheric pressure for 1.5 h. A particular base of formula B is pyridine, or the like.

A compound of formula 7, wherein the variables are as described herein, may be prepared by coupling an activated ester of formula 9, wherein -OL is a leaving group and the

15 other variables are as described herein, with an amino acid/peptide of formula 8, wherein P is as described herein, under appropriate conditions.

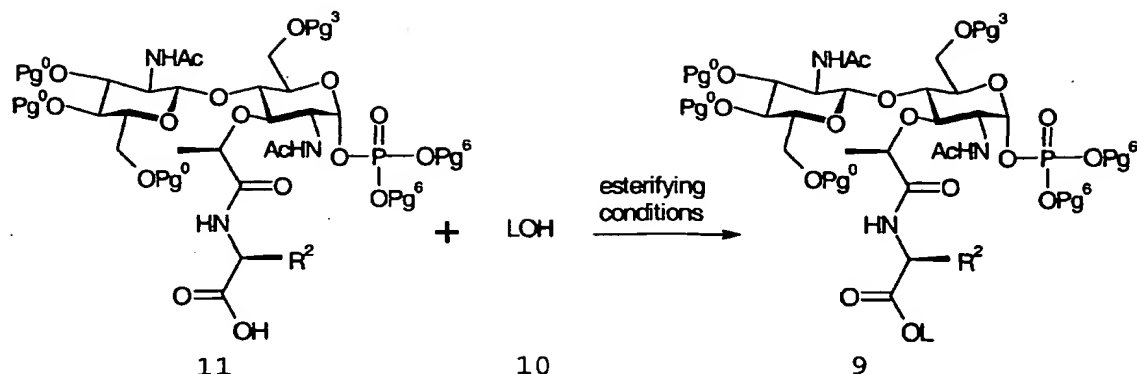


A particular -OL is N-oxysuccinimide, or the like.

Particular conditions encompass dissolving the compounds of
 5 formulas 8 and 9, along with $i\text{Pr}_2\text{NEt}$, or the like, in DMF,
 or the like and stirring at about room temperature under
 argon, or the like for about 18 h.

A compound of formula 8, wherein the variables are as
 described herein, is prepared via conventional peptide
 10 synthetic methods known in the art.

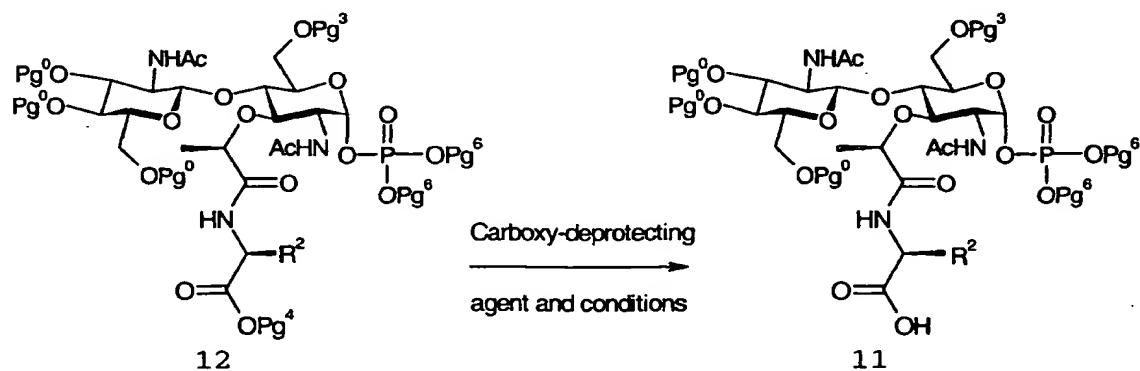
An activated ester of formula 9, wherein the variables
 are as described herein, may be prepared by esterifying an
 acid of formula 11, wherein the variables are as defined
 herein, with a compound of formula 10 under appropriate
 15 conditions.



A particular LOH is N-hydroxysuccinimide, or the like.

Particular conditions encompass dissolving the compound of
 5 formula 11, EDCI, or the like and N-hydroxysuccinimide, or
 the like in anhydrous DMF, or the like and stirring for
 18 h.

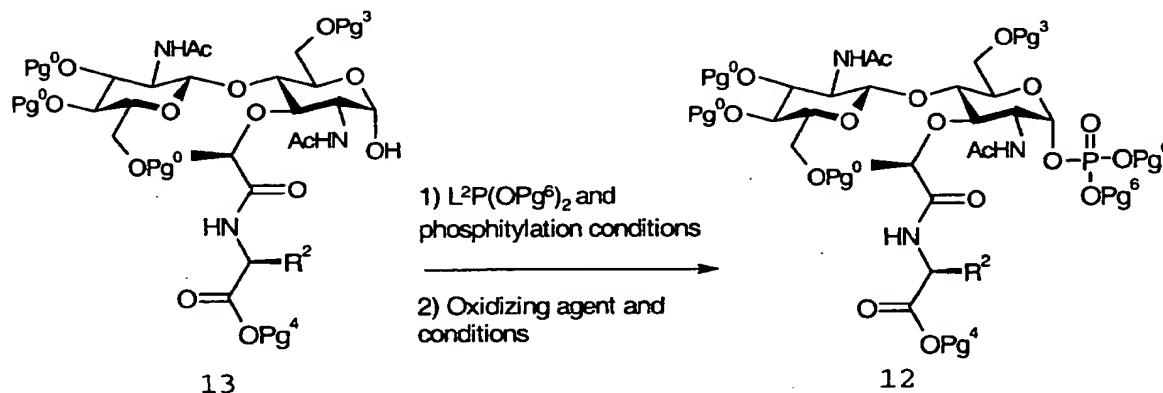
An acid of formula 11, wherein the variables are as
 described herein, may be prepared by removing the Pg⁴ group
 10 of a compound of formula 12, wherein the variables are as
 described herein, in the presence of a carboxy-deprotecting



agent under appropriate conditions. The carboxy-deprotecting
 15 is carried out using an appropriate deprotecting agent that
 depends on the nature of the carboxy-protecting group, i.e.,
 whether it is removable (labile) under acid, base, or

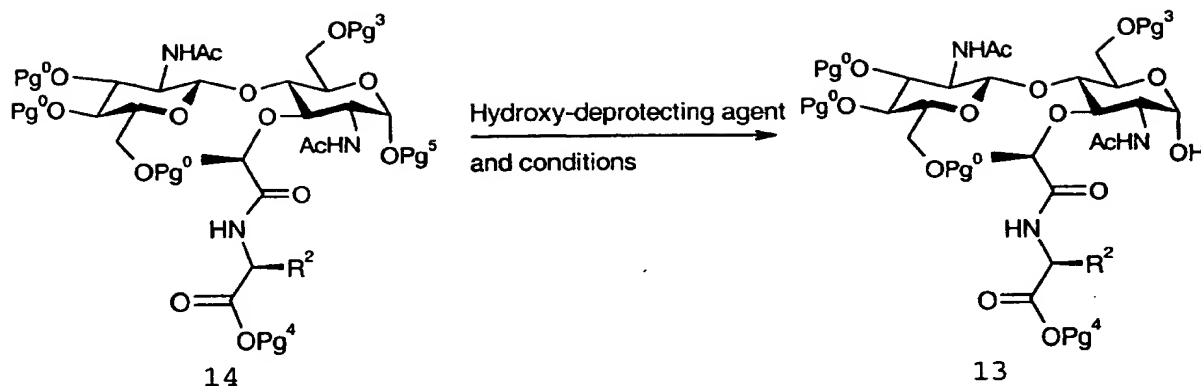
hydrogenation conditions, and other reactive moieties in the compound undergoing deprotection, i.e., a deprotecting agent is chosen to carry out the deprotection without affecting the other reactive moieties unless a concomitant reaction is desired. A particular Pg^4 group is $-\text{CH}_2\text{CH}_2\text{SO}_2\text{Ph}$, or the like. A particular deprotecting agent is DBU, or the like. Particular conditions encompass dissolving compound 12 in CH_2Cl_2 , or the like and adding DBU, or the like dropwise under an argon atmosphere, or the like.

A phosphotriester of formula 12, wherein the variables are as described herein, may be prepared by (1) coupling a lactol of formula 13, wherein the variables are as described herein, with a compound of formula $\text{L}^2\text{P}(\text{OPg}^6)_2$, wherein L^2 is a leaving group and the other variables are as described herein, under appropriate phosphitylation conditions and (2) oxidizing the resulting phosphite to a phosphate in the presence of an oxidizing agent under appropriate oxidizing conditions.



A particular compound of formula $L^2P(OPg^6)_2$ is $Et_2NP(OBn)_2$, or the like. Particular phosphitylation conditions encompass rapidly adding the lactone in anhydrous CH_2Cl_2 , or the like to a vigorously stirred suspension of tetrazole, or the like and $Et_2NP(OBn)_2$, or the like in anhydrous CH_2Cl_2 , or the like under argon, or the like at 25 °C. A particular oxidizing agent is H_2O_2 , or the like. Particular oxidizing conditions encompass dissolving the phosphate in THF and cooling to -80 °C. Then, H_2O_2 is added dropwise to the vigorously stirred solution. After the addition is complete, the ice bath is removed and the mixture allowed to warm to about room temperature over about 2 h. The phosphitylation/coupling sequence is described, for example, in Wong, C.-H, et al., *J. Am. Chem. Soc.* **115**, 2260 (1993); Hitchcock, S. A., et al., *J. Am. Chem. Soc.* **120**, 1916 (1998); and Walker, S., et al., *J. Am. Chem. Soc.* **120**, 2484 (1998), all of which are incorporated herein by reference.

A lactol of formula 13, wherein the variables are as described herein, may be prepared by removing the Pg^5 group of an anomeric benzyl ether of formula 14, wherein the variables are as described herein, in the presence of a hydroxy-protecting agent under appropriate conditions.



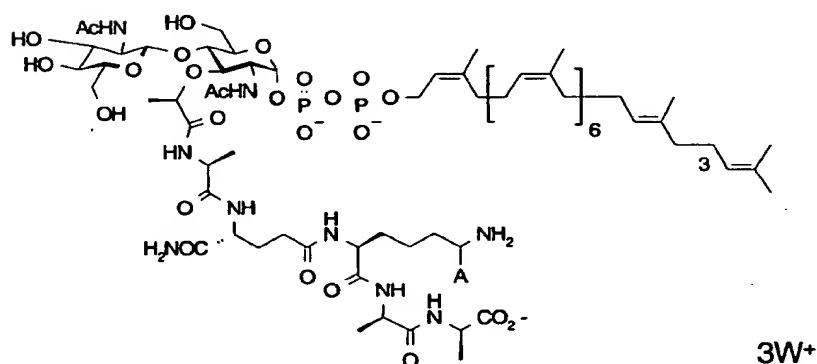
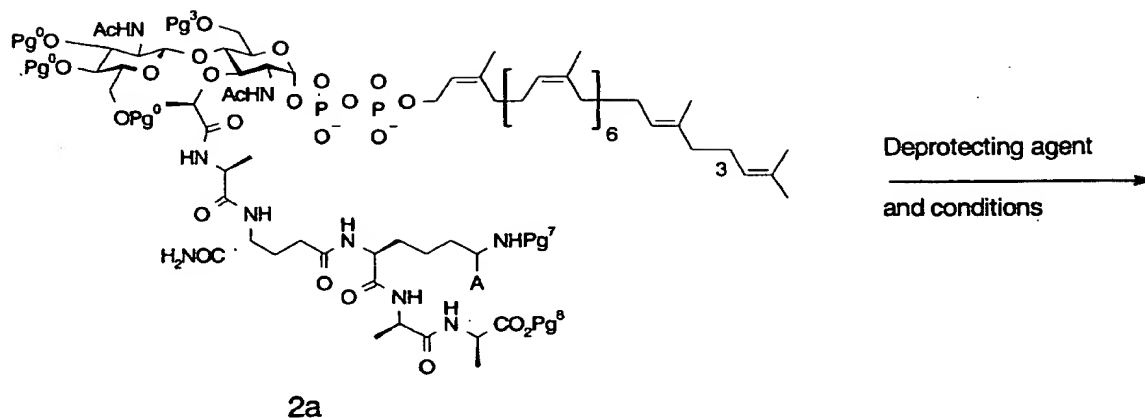
A particular Pg^5 group is benzyl, or the like. A particular hydroxy-deprotecting agent is $\text{H}_2/\text{Pd}/\text{C}$, or the like.

- 5 Particular conditions encompass dissolving the benzyl ether, in HCl /acetic acid, or the like, and adding the resulting solution to a suspension of about 10% Pd/C , or the like in HCl /acetic acid, or the like. The reaction mixture is stirred under about an atmosphere of hydrogen at about 25°C
- 10 for about 1.5 h.

General Preparation of Lipid II

Lipid II, wherein A is as described herein, may be prepared by removing the Pg^0 , Pg^3 , Pg^7 and Pg^8 groups of a

15 compound of formula 2a, wherein the variables are as described herein, in the presence of a



Lipid II

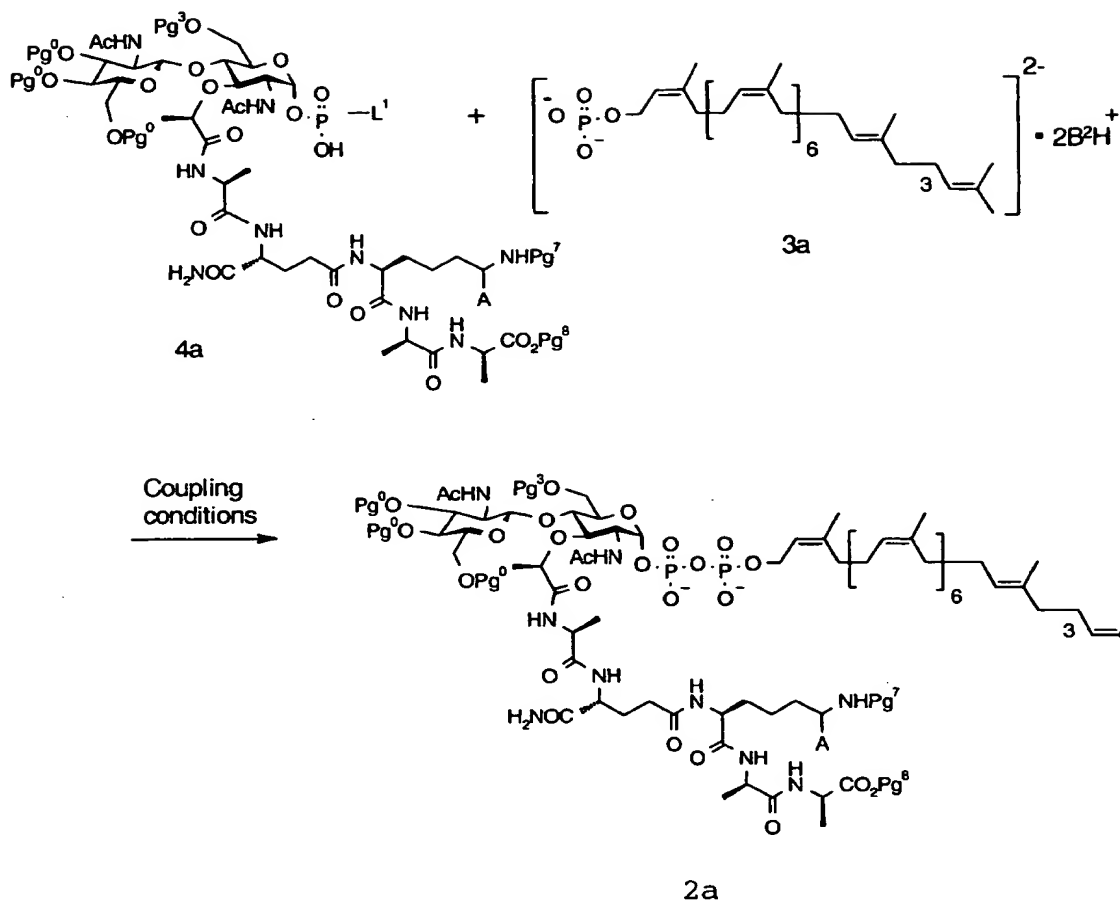
deprotecting agent under appropriate conditions. Particular

5 Pg^0 and Pg^3 groups are acetyl, or the like. A particular Pg^7 group is trifluoroacetyl, or the like. A particular Pg^8 group is methyl, or the like. A particular deprotecting agent is aqueous sodium hydroxide, or the like. Particular conditions encompass carrying out the deprotection in about

10 a 1:1 solution of 1,4-dioxane:water, or the like while stirring for about 2 h. Care must be exercised when exposing the pentapeptide to hydroxide ion mediated deprotection conditions for substantially longer periods of

time since the base-catalyzed rearrangement of iso-glutamine to glutamine in peptidoglycan-related structures has been observed. (see, e.g., Keglevic, D. and A. E. Derome, *Carbohydrate Res.*, **186**, 63 (1989); and Keglevic, D. and J. Kidric, *J. Carbohydrate Res.*, **11**, 119 (1992), incorporated herein by reference).

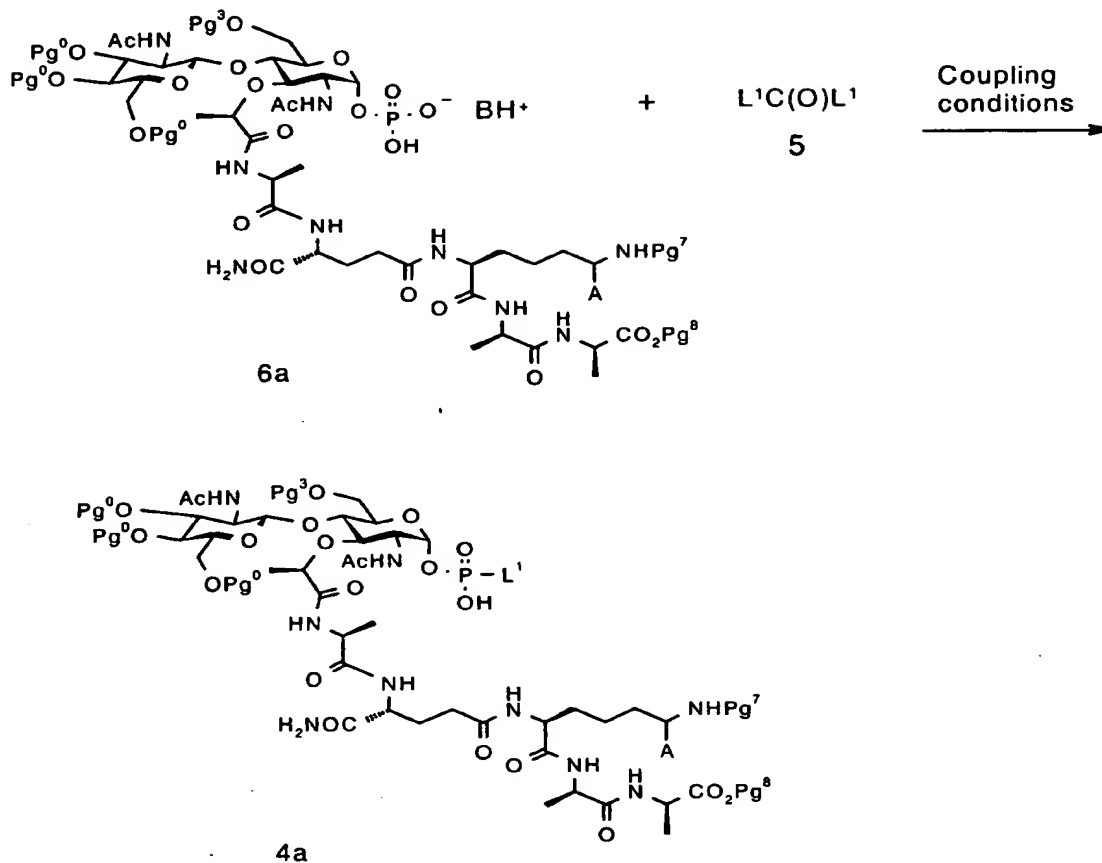
A compound of formula 2a, wherein the variables are as described herein, may be prepared by treating an activated phosphate of formula 4a, wherein the variables are as described herein, with an undecaprenyl monophosphate of formula 3a, wherein B^2 is as described herein, under appropriate coupling conditions.



A particular $-L^1$ is imidazolyl, or the like. A particular compound of formula 3a is undecaprenyl monophosphate (*bis-*
 5 NH_4^+ salt). Particular conditions encompass exposing the activated phosphate to undecaprenyl monophosphate (*bis-* NH_4^+ salt) in the presence of 1*H*-tetrazole, or the like, over about 4 days in DMF/THF, or the like, under an argon atmosphere, or the like.

10 A compound of formula 4a, wherein the variables are as described herein, may be prepared by coupling a compound of formula 6a, wherein the variables are as described herein,

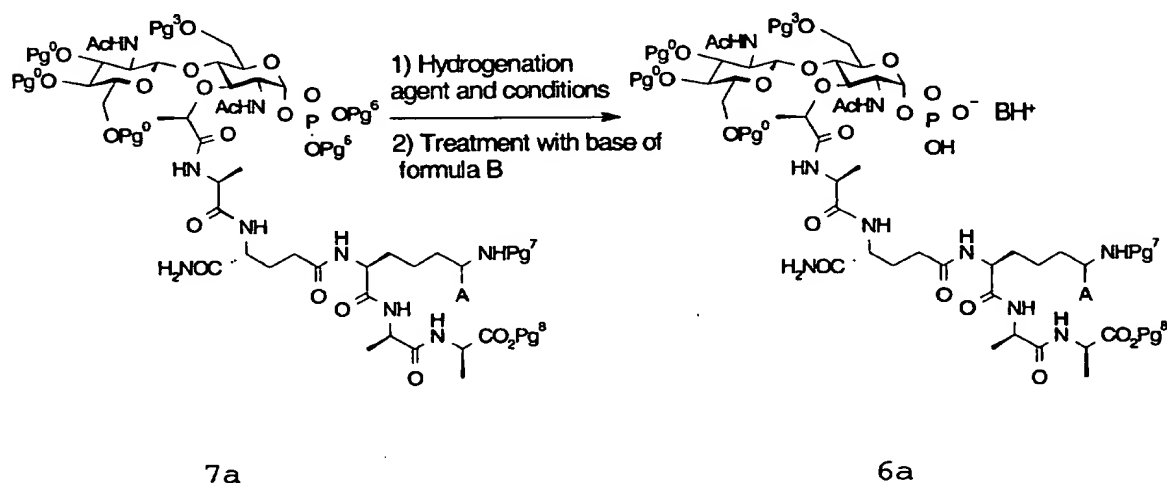
with an activating agent of formula 5 under appropriate conditions.



- 5 The coupling reaction initially forms a phosphate diester intermediate, which gives off CO₂ to yield the activated phosphate of formula 4a. A particular activating agent is CDI, or the like. A particular base counter ion, BH⁺, is C₅H₅NH⁺, or the like. Particular conditions encompass
- 10 carrying out the reaction in anhydrous DMF/THF, or the like, for about 2 h. Electrophilic activation of the anomeric phosphate by conversion to the corresponding

phosphorimidazolate derivative is described in Fang, X., et al., *Bioorg. Med. Chem. Lett.*, **5**, 2701 (1995).

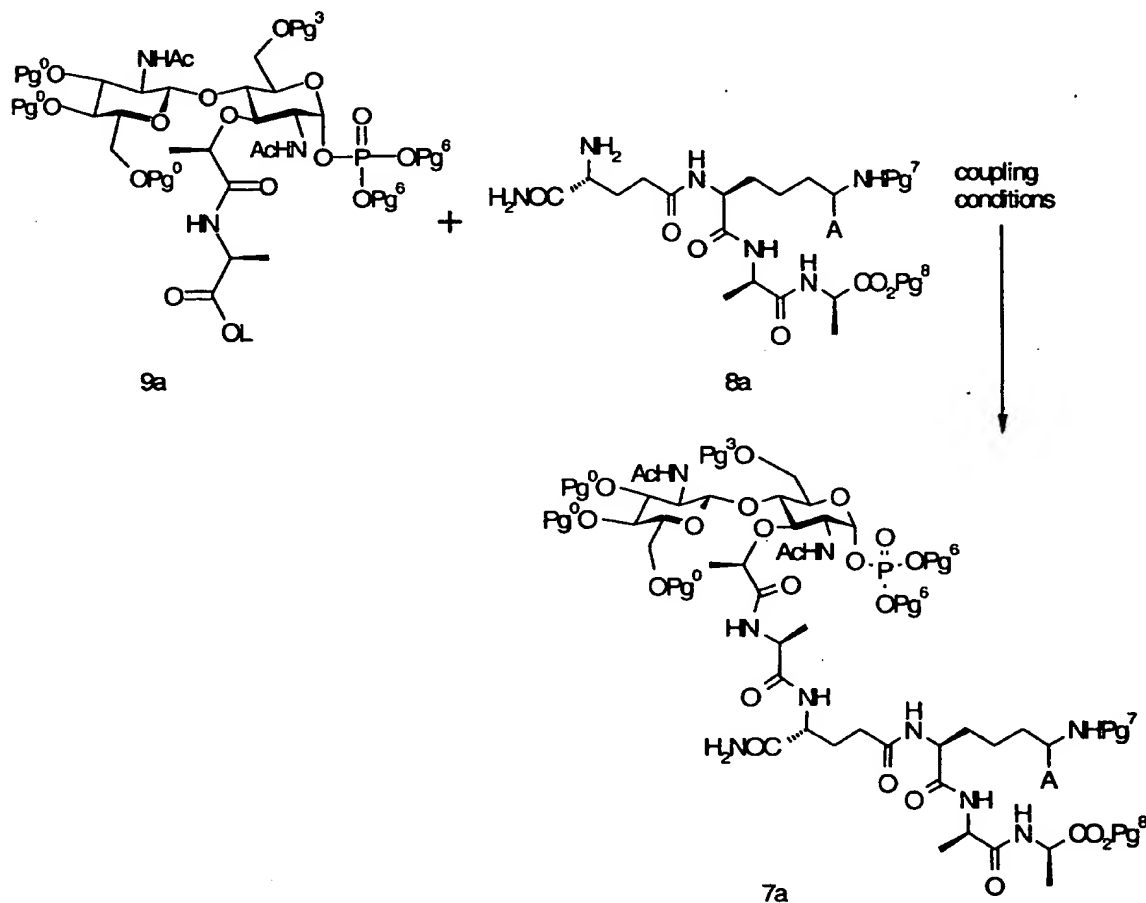
A compound of formula 6a, wherein the variables are as described herein, may be prepared by (1) removing the Pg^6 groups of a compound of compound of formula 7a, wherein the variables are as described herein, in the presence of a hydrogenation agent under appropriate conditions, and (2) treating the resulting compound with a base of formula B to form a phosphate salt.



A particular Pg^6 group is benzyl, or the like. A particular hydrogenation agent is Pd/C, or the like. Particular hydrogenation condition encompasses adding the compound of formula 7a to a suspension of about 10% Pd/C, or the like, in alcohol (preferably methanol), or the like, cooled in an ice bath to aid in degassing the solution. The solution is then warmed to about room temperature and hydrogenated at

about atmospheric pressure for about 1.5 h. A particular base of formula B is pyridine, or the like.

A compound of formula 7a, wherein the variables are as described herein, may be prepared by coupling an activated ester of formula 9a, wherein the variables are as described herein, with a peptide of formula 8a, wherein the variables are as described herein, under appropriate conditions.



A particular -OL group is N-oxysuccinimide, or the like.

Particular conditions encompass dissolving the compounds of formula 8a and 9a along with iPr₂NEt, or the like, in DMF, or the like, and stirring at about room temperature under

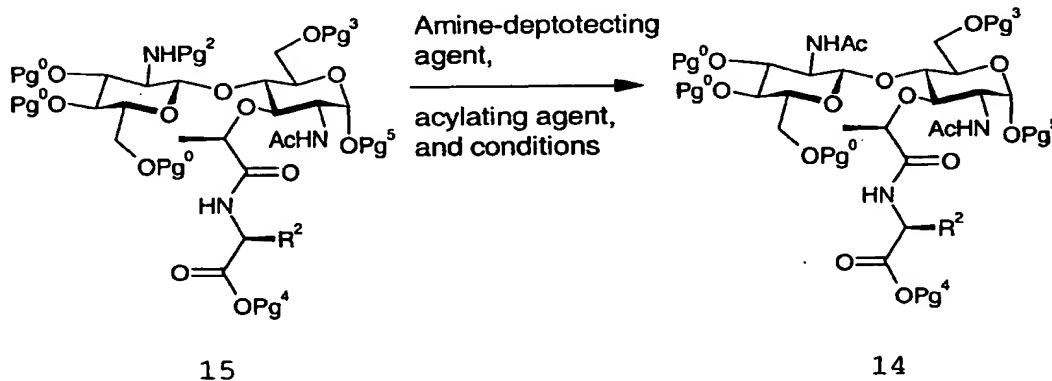
argon, or the like, for about 18 h. There is no evidence for the epimerization of the L-Ala α -stereocenter during the course of the carboxyl activation-peptide coupling sequence.

A compound of formula 8a, wherein the variables are as described herein, is prepared via conventional peptide synthetic methods known in the art.

General Preparation of the NAG-NAM Disaccharide

Starting Material

A compound of formula 14, wherein the variables are as described herein, may be prepared by exchanging the Pg^2 group of a compound of formula 15, wherein the variables are as described herein, with an acetyl group in the presence of



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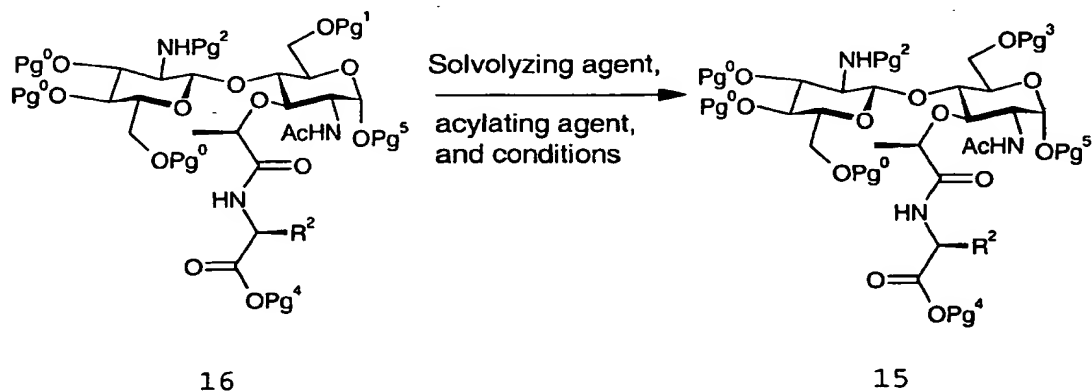
14

an amine-deprotecting agent and an acylating agent under the appropriate conditions. The amine-deprotecting is carried out using an appropriate deprotecting agent that depends on the nature of the amine-protecting group, i.e., whether it is removable (labile) under acid, base, or hydrogenation

conditions, and other reactive moieties in the compound undergoing deprotection, i.e., a deprotecting agent is chosen to carry out the deprotection without affecting the other reactive moieties unless a concomitant reaction is desired. A particular amine-protecting group is 2,2,2-trichloroethoxycarbonyl, or the like. A particular deprotecting agent is Zn dust, or the like, in the presence of a proton source (e.g., acetic acid), or the like. A particular acylating agent is acetic anhydride, or the like.

Particular conditions include adding Zn dust, or the like, and a 3:2:1 mixture of THF:Ac₂O:AcOH to a solution of the compound of formula 8 in about a 2:1 mixture of Ac₂O:AcOH, or the like.

A compound of formula 15, wherein the variables are as described herein, may be prepared by exchanging the Pg¹ group of a compound of formula 16, wherein the variables are as described herein, with a Pg³ group in the presence of a



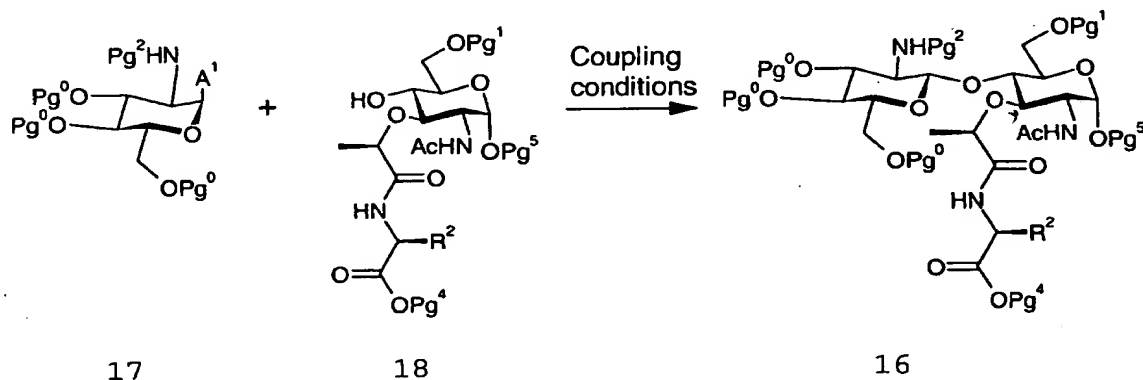
solvolysis agent and acylating agent under appropriate conditions. The solvolysis is carried out using an

appropriate solvolyzing agent that depends on the nature of the hydroxy-protecting group, i.e., a solvolyzing agent is chosen to carry out the solvolysis without affecting the other reactive moieties unless a concomitant reaction is
5 desired. A particular hydroxy-protecting group is benzyl, or the like. A particular solvolyzing agent is ZnCl_2 , or the like. A particular acylating agent is acetic anhydride, or the like. Particular conditions include carrying out the solvolysis/acylation in about a 2:1 mixture of $\text{Ac}_2\text{O}:\text{AcOH}$, or
10 the like.

Synthesis of a compound of formula 16, the central disaccharide core, in orthogonally protected form presents a significant synthetic challenge. For example, identification of a protective scheme having triple
15 orthogonality is highly desirable to accomplish selective unmasking of the three types of pendant hydroxyl groups (i.e., anomeric OH, peripheral OH, and carboxyl OH). In addition, the stereoselective construction of the β -[1,4] glycosidic linkage is expected to be difficult irrespective
20 of the method used to generate a reactive glycosyl cation donor. For example, with respect to the glycosyl cation, each of the following inherent properties contribute to a loss of reactivity of the glycosyl cation acceptor: (i) the intrinsic lack of nucleophilicity of the C(4)-hydroxyl group
25 of glucopyranose-based acceptors, (ii) additional steric crowding around the C(4)-hydroxyl of muramic acid-based

acceptors, and (iii) additional electronic deactivation of 2-deoxy-2-acylamino-glucopyranose acceptors relative to their glucopyranose-based counterparts. With respect to the glycosyl cation donor, an activation method with a
 5 predisposition toward formation of a β -[1,4] glycosidic linkage is needed. The reaction conditions for glycosyl cation generation also needs to be compatible with functionality resident in both the donor and acceptor.

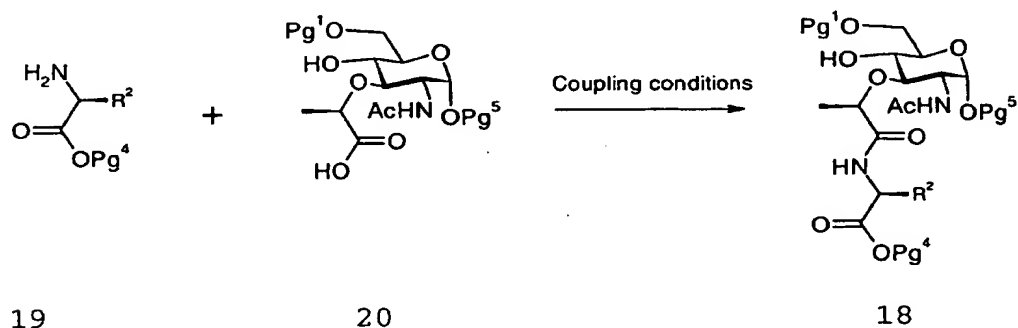
A compound of formula 16, wherein the variables are as
 10 described herein, may be prepared by coupling a muramylamide compound of formula 17, wherein A^1 is Br or Cl (preferably Br) and the other variables are as described herein, with a



15 glucopyranosyl compound of formula 17, wherein the variables are as described herein, under appropriate conditions. Particular conditions include carrying out the coupling reaction under scrupulously anhydrous Konigs-Knorr conditions (e.g., in a silver triflate/dichloromethane
 20 solution including molecular sieves), or the like.

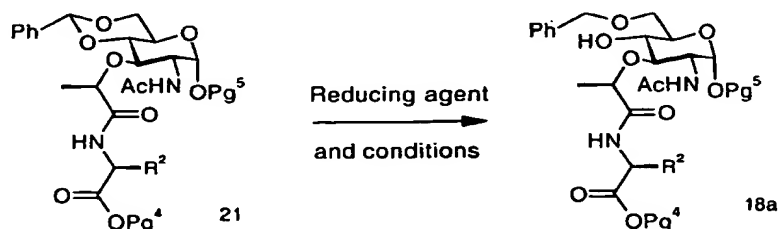
A compound of formula 17 is prepared according to the procedures described in Imoto, M., *Bull. Chem. Soc. Jpn.*, **60**, 2205 (1987).

A compound of formula 18, wherein the variables are as described herein, may be prepared by coupling an acid of formula 20, wherein the variables are as described herein,



with a protected amino acid/peptide compound of formula 19, wherein the variables are described herein, under appropriate conditions. Particular conditions encompass carrying out the coupling reaction in a solution of NMM, or the like, and 2-chloro-4,6-dimethoxy-1,3,5-triazine, or the like, in CH₂Cl₂, or the like, wherein the compound of formula 19 is added as the tosylate salt, or the like.

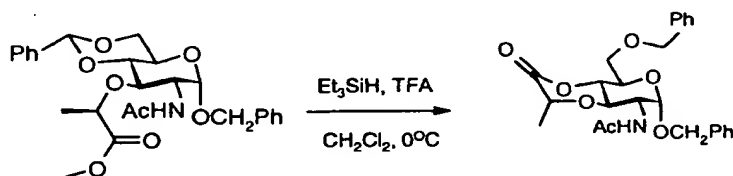
A compound of formula 18a, wherein Pg¹ is benzyl and the other variables are as described herein, may be prepared by treating a compound of formula 21, wherein the variables



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are as defined herein, with a reducing agent under appropriate conditions. A particular reducing agent is triethylsilane, or the like. Particular conditions include carrying out the reduction in CH_2Cl_2 or the like, and TFA, 5 or the like, at about 0°C . This reaction provides an efficient means for regioselective introduction of the benzyl protection/activation at C(6)OH of the muramic acid derivative.

It is known that when an ester derivative of a compound 10 of formula 21 is treated with trifluoroacetic acid and triethylsilane as described in DeNinno, M.P., et al., *Tetrahedron Lett.*, **36**, 669 (1995), only a small amount of the analogous compound of formula 18a is observed. The major product formed is a lactone, as shown in scheme I.

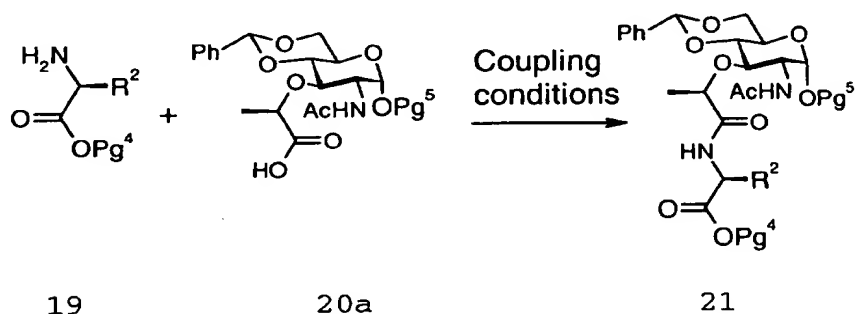


Scheme I

The acid-catalyzed lactonization proceeds at a rate competitive with the reductive ring opening, thus leading to the undesired lactone. In the present invention, however, 20 introduction of an amide bond in place of the ester bond eliminates the conversion to the lactone, thus allowing the

desired product (compound 18a) to be isolated in much higher yields.

A compound of formula 21, wherein the variables are as described herein, may be prepared by coupling an acid of
5 formula 20a, wherein the variables are as described herein,



with a protected amino acid of formula 19, wherein the
10 variables are described herein, under appropriate
conditions. Particular conditions encompass carrying out
the coupling reaction in a solution of NMM, or the like, and
2-chloro-4,6-dimethoxy-1,3,5-triazine, or the like, in
CH₂Cl₂, or the like, wherein the protected amino acid is
15 added as the tosylate salt, or the like.

General Experimental

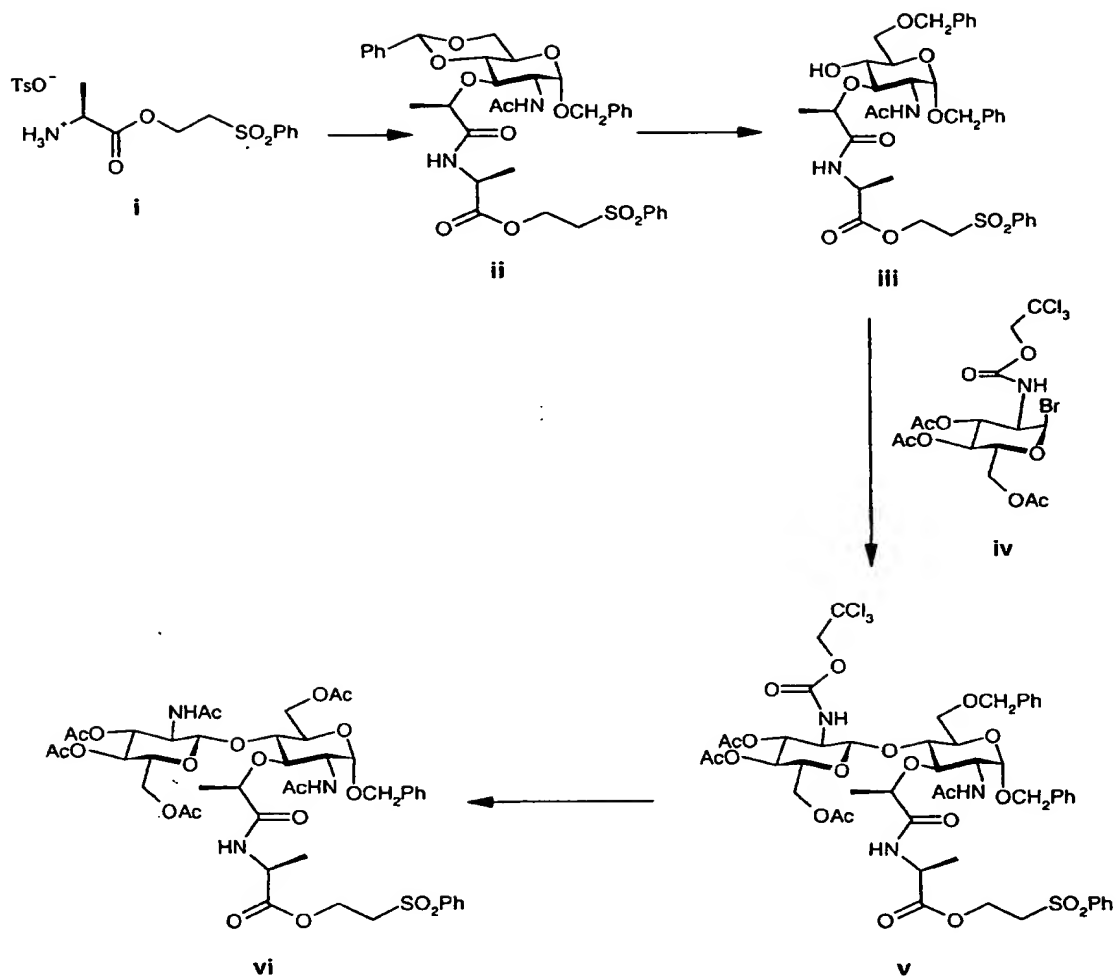
Reactions are carried out with continuous stirring under a positive pressure of nitrogen except where noted.

20 Reagents and solvents are purchased and used without further purification. TLC is performed using 0.25 mm silica gel 60 plates with a 254 nm fluorescent indicator from E. Merck.

Plates are developed in a covered chamber and visualized by ultraviolet light or by treatment with 5% phosphomolybdic acid (or alternatively CAM) in ethanol followed by heating. Flash chromatography is carried out with silica gel 60, 230-
5 400 mesh (0.040-0.063 mm particle size) purchased from EM Science. HPLC analyses and purifications are performed using Dynamax C8 columns with the specified solvent system and flow rate. NMR spectra are reported as chemical shifts in parts-per-million (ppm) downfield from a
10 tetramethylsilane internal standard (0 ppm). ^1H NMR spectra are recorded in the solvent indicated on either a Bruker Avance spectrometer at 500.18 MHz, a Varian Mercury spectrometer at 400.21 MHz, or a GE QE-300 spectrometer at 300.15 MHz. ^{13}C NMR spectra are recorded in the solvents
15 indicated on the previously mentioned spectrometers at 125.78 Mz, 100.15 MHz, and 75.48 MHz, respectively. IR spectra are recorded on a Nicolet 510P FT-IR spectrometer; electrospray ionization mass spectra (ESI-MS) are recorded on a Micromass Platform LCZ spectrometer. High resolution
20 mass spectra are recorded on a Micromass QTOF mass spectrometer.

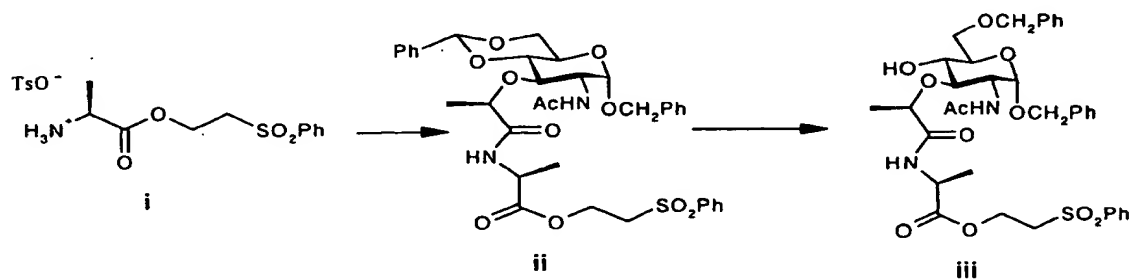
Preparation of the NAG-NAM Disaccharide Starting Material

A synthetic process for preparing the NAG-NAM
25 disaccharide starting material is outlined in Scheme II and exemplified below.



Scheme II

5 I. Regioselective Installation of Benzyl Protection & Attachment of Peptide Linker:



A mixture of (L)-alanine (15.0 g, 168 mmol), phenylsulfonyl ethanol (37.6 g, 202 mmol), and TsOH·H₂O (35.2 g, 185 mmol) in benzene (750 mL) is refluxed using a Dean-Stark apparatus. After 16 h, additional phenylsulfonyl ethanol (25 g, 135 mmol) and TsOH·H₂O (25 g, 134 mmol) is added along with benzene (180 mL), and the reaction mixture is refluxed overnight. Concentration *in vacuo* gives the product, **compound i**, in quantitative yield as a white solid.

Analytical (compound i): ¹H NMR(DMSO-*d*₆, 300 MHz) δ 8.25(br s, impurities, TsOH), 7.94-7.88(m, 2H), 7.81-7.74(q, *J* = 6.2 Hz, 1H), 7.71-7.62(m, 2H), 7.49(d, *J* = 8.1 Hz, 1H), 7.12(d, *J* = 8.1 Hz, 1H), 5.39(br s, 2H), 4.52-4.44(m, 1H), 4.41-4.33(m, 1H), 3.90-3.82(m, 1H), 3.78(t, *J* = 5.5 Hz, 2H), 3.67(t, *J* = 6.2 Hz, 1H), 3.44(t, *J* = 6.6 Hz, 1H), 2.29(s, 3H + impurities, TsOH), 1.20(d, *J* = 7.3 Hz, 3H) ¹³C NMR (DMSO-*d*₆, 75 MHz) δ 169.5, 145.5, 139.3, 137.7, 134.1, 133.6, 129.5, 129.3, 128.0, 127.7, 127.6, 125.5, 58.9, 57.5, 54.9, 53.6, 47.7, 20.7, 15.2; MS(ESI) *m/z* 258.1 (100%, M-TsOH-H); IR KBr) *v*_{max} 3424(br), 2927(br), 1745(m), 1309(m), 1224(m), 1195(m), 1147(s), 1124(m), 1087(m), 1007(m) cm⁻¹; Anal. Calcd for C₁₈H₂₅NO₄S: C, 50.10; H, 5.84; N, 3.25; S, 14.86. Found: C, 48.49; H, 5.31; N, 2.56; S, 14.72.

To a slurry of benzyl *N*-acetyl-4,6-benzylidene muramic acid (20.0 g, 42.5 mmol) in CH₂Cl₂ (300 mL) at 0 °C is added

N-methylmorpholine (NMM) (4.67 mL, 42.5 mmol) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (8.94 g, 51.0 mmol). After stirring for 45 min at 0°C, CH₂Cl₂ (300 mL) followed by NMM (9.34 mL, 83.0 mmol) and L-alanine(phenylsulfonylethyl ester, tosylate salt) (15.4 g, 51.0 mmol) (i.e., **compound i**) are added to the above reaction mixture. The resulting solution is slowly warmed to room temperature and stirred for 3 days. The reaction mixture is then filtered. The filtrate is washed first with 1N HCl then with brine, and dried (MgSO₄). The filtrate is then concentrated under reduced pressure, evaporated with toluene(x2), and vacuum dried overnight to afford the product, **compound ii**, (23.5 g, 95%) as a white solid.

Analytical (**compound ii**): ¹H NMR (CDCl₃, 400 MHz) δ

7.44(d, *J* = 3.0 Hz, 2H), 7.35(m, 8H), 6.95(d, *J* = 6Hz, 1H), 6.15(d, *J* = 6.0 Hz, 1H), 5.85(m, 1H), 5.47(s, 1H), 5.21(dd, *J* = 3.0, 12.0 Hz, 1H), 5.30(dd, *J* = 3.0, 15.0 Hz, 1H), 4.90(d, *J* = 3.0 Hz, 1H), 4.72(d, *J* = 12.0 Hz, 1H), 4.60(m, 2H), 4.42(m, 2H), 4.30-4.20(m, 2H), 4.15(q, *J* = 3.0 Hz, 1H), 4.00(q, *J* = 3.0 Hz, 1H), 3.82(m, 1H), 3.75(d, *J* = 9.0 Hz, 1H), 3.65(m, 1H), 1.93(s, 3H), 1.43(d, *J* = 3.0, 9.0 Hz, 3H), 1.38(d, *J* = 3.0, 9.0 Hz, 3H); ¹³C NMR (CDCl₃, 75 MHz) δ

173.2, 172.2, 170.6, 131.6, 129.0, 128.9, 128.4, 128.3, 125.9, 101.4, 97.5, 81.7, 78.3, 76.6, 75.6, 75.1, 70.1, 68.9, 65.8, 64.1, 63.2, 55.3, 53.2, 48.1, 23.0, 17.4, 17.8.

MS (ESI) m/z 583.2 (86%, M+H), 581.3 (100%, M-H); IR ν_{\max} (CHCl₃) 3010(m), 1740(m), 1681(s), 1616(m), 1569(s), 1523(m), 1470(m), 1377(s), 1333(m), 1119(m), 1090(m) cm⁻¹; Anal. Calcd for C₃₁H₃₈N₂O₉: C, 63.90; H, 6.57; N, 4.81.

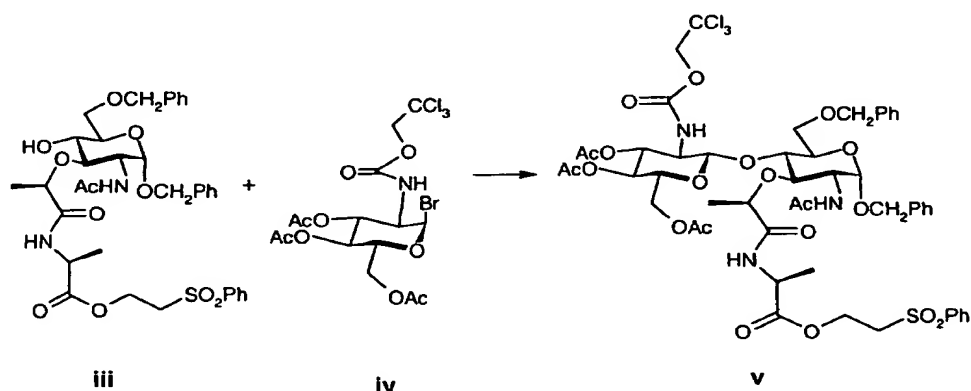
5 Found: C, 63.78; H, 6.55; N, 4.89.

Triethylsilane (16.4 mL, 103 mmol) is added to a solution of **compound ii** (12.0 g, 20.6 mmol) in CH₂Cl₂ (150 mL) at 0°C, followed by dropwise addition of TFA (8.1 mL, 103 mmol). The mixture is allowed to stir for 5 h, after
10 which an additional 3 equivalents of TFA (5.0 mL) is added dropwise, and stirred at 0°C overnight. Upon completion of the reaction, as evidenced by TLC (EtOAc), the reaction mixture is diluted with CH₂Cl₂, then NaHCO₃ is added slowly to neutralize the TFA. The aqueous layer is extracted with
15 CH₂Cl₂. The organic layer is washed with brine (x2), then dried (MgSO₄), and concentrated *in vacuo*. Purification by prep-LC (eluting with 70:30 EtOAc:hexane to EtOAc), followed by recrystallization from CH₂Cl₂ and isopropyl ether gives the product, **compound iii**, (7.4 g, 61%) as a white solid.

20 Analytical (**compound iii**): ¹H NMR (CDCl₃, 300 MHz) δ 7.38-7.26(m, 10H), 6.99 d, J = 7.3 Hz, 1H), 6.16(d, J = 8.8 Hz, 1H), 5.94-5.81(m, 1H), 5.30(dd, J = 1.1, 17.2 Hz, 1H), 5.22(dd, J = 1.1, 10.6 Hz, 1H), 4.92(d, J = 3.7 Hz, 2H), 4.68(t, J = 11.7 Hz, 1H), 4.59(d, J = 7.7 Hz, 4H), 4.49(q, J
25 = 6.2 Hz, 1H), 4.46(dd, J = 2.2, 11.7 Hz, 2H), 4.21(dq, J =

3.7, 9.9 Hz, 1H), 4.17(q, $J = 7.0$ Hz, 1H), 3.83-3.75(m, 1H),
 3.71(t, $J = 5.1$ Hz, 1H), 3.68-3.65(m, 1H), 3.54(t, $J = 10.2$
 Hz, 1H), 1.89(s, 3H), 1.44(d, $J = 7.0$ Hz, 3H), 1.40(d, $J =$
 7.0 Hz, 3H); ^{13}C NMR(CDCl_3 , 75 MHz) δ 173.0, 172.3, 170.3,
 5 167.7, 137.8, 137.1, 131.7, 128.6, 128.5, 128.1, 127.8,
 127.7, 118.5, 97.1, 80.5, 77.7, 73.7, 71.6, 70.5, 70.2,
 69.8, 65.8, 55.1, 52.5, 48.0, 24.5, 23.3, 19.2, 17.7;
 MS(ESI) m/z 585.2 (100%, $M+H$), 583.2 (100%, $M-H$); IR
 ν_{max} (CHCl_3) 3433(m), 3010(m), 1741(m), 1677(s), 1522(m),
 10 1454(m), 1124(m), 1058(m) cm^{-1} ; Anal. Calcd for $\text{C}_{31}\text{H}_{40}\text{N}_2\text{O}_9$:
 C, 63.68; H, 6.90; N, 4.79; Found: C, 63.67; H, 6.58; N,
 4.83.

II. Glycosidation



15

Compound iv is prepared using the procedures described
 in Imoto, M., *Bull. Chem. Soc. Jpn.*, **60**, 2205 (1987).

To a solution of **compound iii** (4.59 g, 6.43 mmol) in
 CH_2Cl_2 (30 mL) are added 4Å molecular sieves (10 g) and

silver triflate (5.12 g, 20.0 mmol). To this mixture is added a solution of freshly prepared **compound iv** (10.8 g, 20.0 mmol) in CH₂Cl₂ (9.5 mL) in four portions over a 1 h period. Each of the starting materials is dried prior to use, and the reaction is performed under controlled anhydrous conditions. After stirring at room temperature for 24 h, the reaction mixture is filtered through Celite and washed with CH₂Cl₂. The organic layer is washed with NaHCO₃, brine, dried (Na₂SO₄), and concentrated in vacuo.

10 Purification by column chromatography on silica (Flash Elute system) utilizing a solvent gradient of 50% hexane in EtOAc, 15% hexane in EtOAc, EtOAc, and 5% MeOH in EtOAc yields the product, **compound v**, (5.73 g, 76%) as a white solid, along with unreacted starting material, **compound iii**, (630 mg, 14%).

15

Analytical (compound v): ¹H NMR(CDCl₃, 300 MHz)

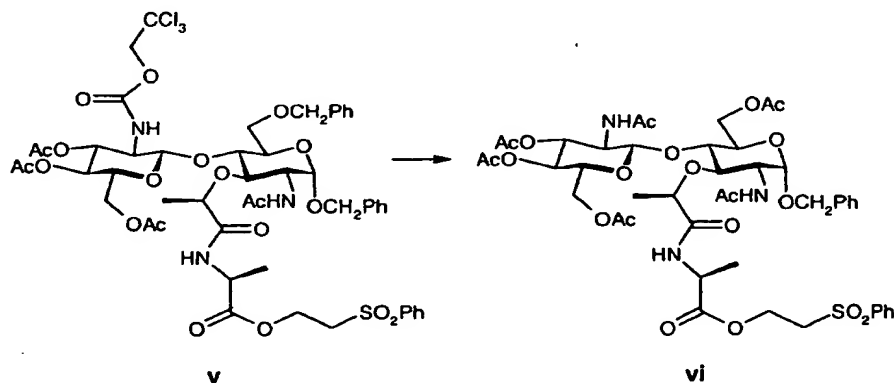
87.91(d, *J* = 7.0 Hz, 2H), 7.66(t, *J* = 7.3 Hz, 1H), 7.58-7.50(m, 4H), 7.45(t, *J* = 7.3 Hz, 2H), 7.33-7.26(m, 6H), 6.83(d, *J* = 7.3 Hz, 1H), 6.52(d, *J* = 7.0 Hz, 1H), 5.09(d, *J* = 2.9 Hz, 1H), 4.97(t, *J* = 9.5 Hz, 1H), 4.87(d, *J* = 12.1 Hz, 1H), 4.79-4.73(m, 2H), 4.60(dd, *J* = 7.3, 12.1 Hz, 2H), 4.53-4.29(m, 5H), 4.26-4.04(m, 7H), 4.00-3.88(m, 2H), 3.70-3.50(m, 4H), 3.42(t, *J* = 10.6 Hz, 4H), 2.03(s, 3H), 1.98(s, 6H), 1.89(s, 3H), 1.34(d, *J* = 6.6 Hz, 3H), 1.24(d, *J* = 7.3 Hz, 3H); ¹³C NMR(CDCl₃, 75 MHz) δ173.4, 171.8, 170.6, 170.3,

20

25

169.4, 154.1, 137.3, 134.0, 129.4, 129.1, 128.5, 128.1,
 100.0, 97.1, 96.9, 77.4, 77.0, 76.6, 75.7, 74.5, 73.8, 72.2,
 71.2, 70.4, 70.3, 68.3, 67.2, 61.5, 58.1, 26.2, 54.9, 53.6,
 47.7, 23.2, 20.6, 18.3, 17.5; MS (FAB) m/z 1176.3 (73%,
 5 M+H), (ESI) m/z 1174.5 (62%, M-H) IR (KBr) ν_{\max} 3385(br),
 3067(w), 2939(w), 1753(s), 1669(m), 1537(m), 1233(s),
 1145(m), 1045(s) cm^{-1} ; UV-vis (95% EtOH) λ_{\max} 264
 (1223.11)nm; Anal. Calcd for $\text{C}_{51}\text{H}_{64}\text{Cl}_3\text{N}_3\text{O}_{20}\text{S}$: C, 52.02; H,
 5.48; N, 3.57; S, 2.72; Cl, 9.03. Found: C, 51.72; H, 5.40;
 10 N, 3.64; S, 2.72; Cl, 9.07.

III. Protective Group Interchange



15

To a solution of **compound v** (1.9 g, 1.57 mmol) in
 $\text{Ac}_2\text{O}:\text{AcOH}$ (2:1, 11 mL) is added a solution of ZnCl_2 (2.1 g,
 15.7 mmol) in $\text{Ac}_2\text{O}:\text{AcOH}$ (2:1, 5 mL) in one portion. Upon
 completion of the reaction (24 h) as judged by TLC (EtOAc),
 20 Troc is removed by adding Zn dust (4.1 g, 62.8 mmol) and a
 mixture of $\text{THF}:\text{Ac}_2\text{O}:\text{AcOH}$ (3:2:1, 25 mL) to the above

reaction mixture and stirring until no starting material is evidenced by TLC (EtOAc). The reaction mixture is filtered through Celite, washed with EtOAc, and then concentrated under reduced pressure. The residue is repeatedly
5 evaporated with toluene to remove any remaining Ac₂O and AcOH, and then diluted with EtOAc. The organic layer is washed with NaHCO₃ (x2), H₂O (x2), and brine. The organic layer is then dried (Na₂SO₄) and concentrated *in vacuo*. Purification via column chromatography on silica (Flash
10 Elute system) eluting with 2% MeOH in EtOAc affords the product, **compound vi**, (1.0 g, 67%) as a white solid.

Analytical (compound vi): ¹H NMR (CDCl₃, 300 MHz) δ
7.89(d, *J* = 7.0 Hz, 2H), 7.66(t, *J* = 7.3 Hz, 1H), 7.56(t, *J*
= 7.7 Hz, 2H), 7.34-7.23(m, 6H), 7.16(d, *J* = 7.7 Hz, 1H),
15 6.88(d, *J* = 7.0 Hz, 1H), 6.12(d, *J* = 9.5 Hz, 1H), 5.12-
5.07(m, 3H), 4.56 (dd, *J* = 12.1, 40.0 Hz, 2H), 4.45(d, *J* =
9.0 Hz, 1H), 4.39(d, *J* = 8.4 Hz, 1H), 4.35-4.23(m, 4H),
4.17(d, *J* = 12.0 Hz, 2H), 4.09-3.95(m, 3H), 3.78(d, *J* = 5.5
Hz, 2H), 3.60-3.48(m, 3H), 3.41-3.30(m, 2H), 2.12(s, 3H),
20 2.01(s, 3H), 2.00(s, 3H), 1.99(s, 3H), 1.94(s, 3H), 1.92(s,
3H), 1.38 (d, *J* = 6.6 Hz, 3H), 1.28(d, *J* = 7.3 Hz, 3H); ¹³C
NMR(CDCl₃, 75 MHz) δ173.8, 171.9, 171.2, 170.9, 170.8,
170.6, 169.3, 139.2, 137.3, 134.1, 129.4, 128.9, 128.5,
128.1, 128.0, 127.8, 100.2, 96.9, 77.1, 76.6, 75.9, 75.6,
25 72.5, 71.8, 70.2, 69.5, 68.2, 62.3, 61.6, 58.0, 54.9, 54.6,

5

I. Preparation of Cbz-D-iGln (compound ix)



15

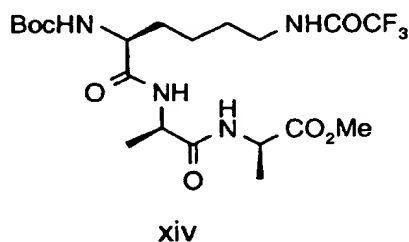
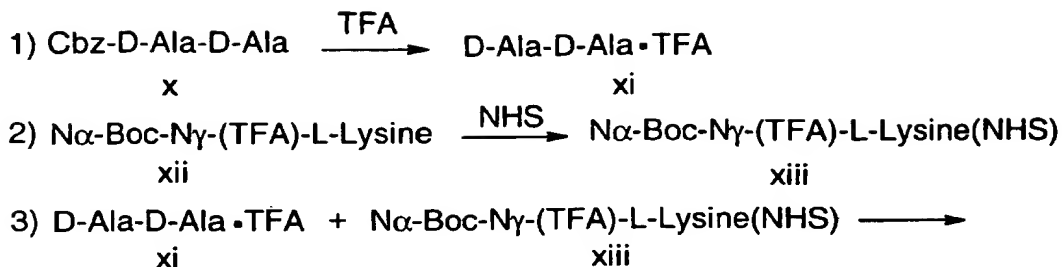
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Analytical (compound viii): mp 134-135°C. IR(CHCl₃) 2985, 1717, 1695, 1155 cm⁻¹; ¹H NMR(DMSO-d₆, 300 MHz) δ 7.35(s, 5H), 7.30(brs, 1H), 5.02(s, 2H), 3.94(m, 1H), 2.22(t, J = 7.7 Hz, 2H), 1.86(m, 1H), 1.69(m, 1H), 1.38(s, 9H); MS (ESI) m/z 337 (80%, M+H); Anal. Calcd for C₁₇H₂₄N₂O₅: C, 60.70; H, 7.19; N, 8.33. Found C, 60.41; H, 7.15; N, 8.16.

Trifluoroacetic acid (25 mL) is added to a solution of Cbz-D-iGln(O^tBu) (3.20 g, 9.52 mmol of **compound viii**) in methylene chloride (25 mL) and the mixture is stirred for 3 h under N₂. The solution is evaporated to give a white solid, filtered with the aid of ethyl ether, and dried in high vacuum to provide homogeneous Cbz-D-iGln (2.2 g, 82% yield of **compound ix**).

Analytical (compound ix): mp 174-176°C. IR (KBr) 3314, 1699, 1658, 1254 cm⁻¹; ¹H NMR(DMSO-d₆, 300 MHz) δ 7.35(s, 5H), 7.31(s, 1H), 7.01(s, 1H), 5.02(s, 2H), 3.93(m, 1H), 2.25(t, J = 8.0 Hz, 2H), 1.92(m, 1H), 1.73(m, 1H); MS (ESI) m/z 281 (100%, M+H); Anal. Calcd for C₁₃H₁₆N₂O₅ • 0.25 H₂O; C, 54.83; H, 5.79; N, 9.84. Found C, 55.12; H, 5.53; N, 9.88.

II. Preparation of N α -Boc-N γ -(TFA)-L-Lys-D-Ala-D-Ala-OMe (compound xvi)



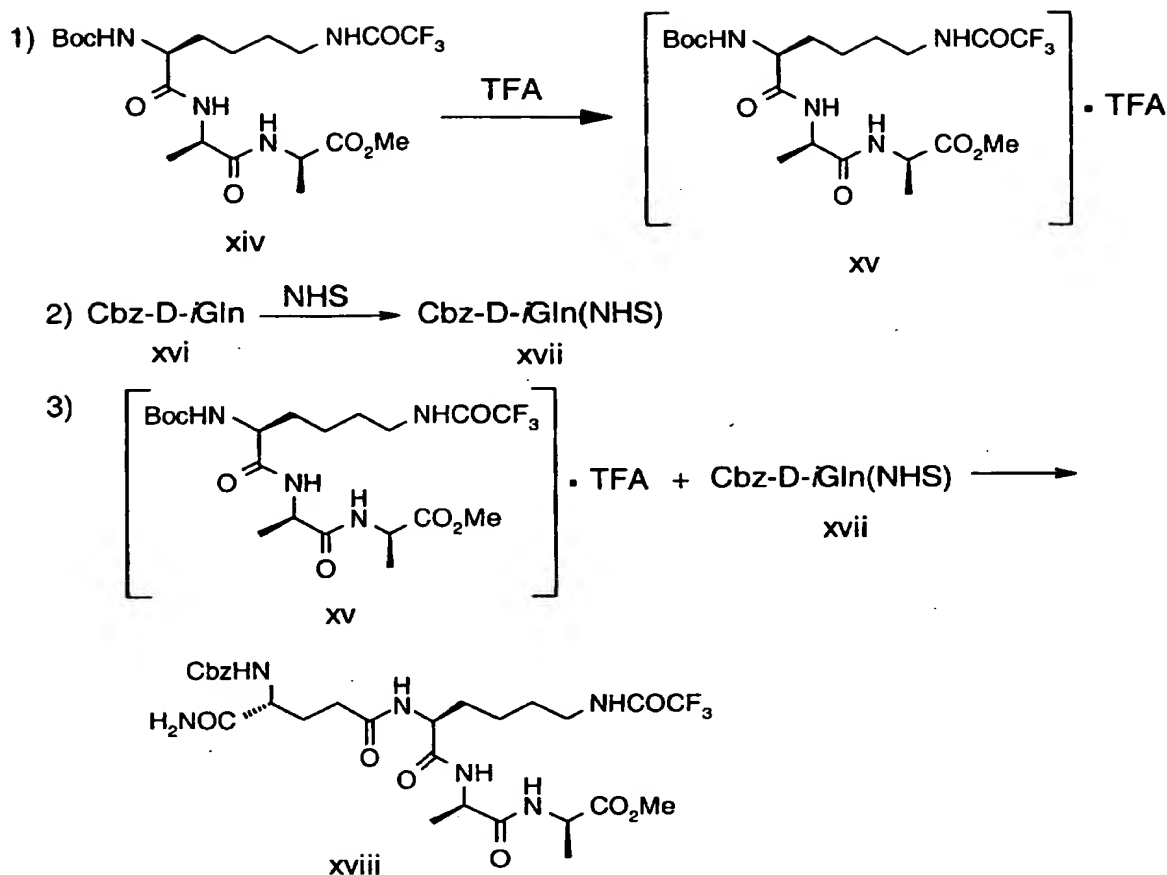
Cbz-D-Ala-D-Ala (1.20 g, 3.9 mM of **compound x**, available from Advanced Chem Tech., Louisville, KY) is dissolved in 10 mL methanol and added to a suspension of 500 mg Pd(OH)₂ in 15 mL methanol containing 0.3 mL TFA (3.9 mM). The mixture is stirred vigorously under one atmosphere of hydrogen (balloon pressure) for 2 h, filtered through talc, concentrated *in vacuo*, and the resulting **compound xi** (the TFA salt of D-Ala-D-Ala) stored under vacuum. Separately, N α -Boc-N γ -(TFA)-L-Lysine (1.40 g, 4 mM of **compound xii**, available from Bachem, Torrance, CA) and 830 mg DCC (4.0 mM) are dissolved in 15 mL dry THF and stirred at 0°C for 3 h. N-hydroxysuccinimide (460 mg, 4.0 mM) is then added, after which the flask is allowed to warm to room temperature and stirred for an additional hour to give **compound xiii**, the NHS-activated ester of **compound xii**. The reaction mixture is then filtered directly into a flask containing **compound**

-72-

xi, followed by the addition of 1.4 mL diisopropylethylamine (0.8 mM). After 30 minutes the reaction mixture is concentrated in vacuo, dissolved in ethyl acetate, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄,
5 and then concentrated in vacuo to a white foam (1.4 g, 2.8 mM, 72% yield of **compound xiv**). The isolated material was used in the next step without further purification.

Analytical (**compound xiv**): ¹H(CDCl₃): δ 1.37(d, 3H, J = 7.5 Hz), 1.39(d, 3H, J = 7.2 Hz), 1.4(s, 9H), 1.75(m, 6H),
10 3.35(t, 2H, J = 6.3 Hz), 3.75(s, 3H), 4.15(m, 1H), 4.48(m, 2H), 5.1(d, 1H), 6.67(d, 1H), 6.78(d, 1H). MS(FD⁺): 499.
EA calc. for C₂₀H₃₃F₃N₄O₇; C, 48.2; H, 6.7; N, 11.2 Found: C, 47.9; H, 6.5; N, 11.1.

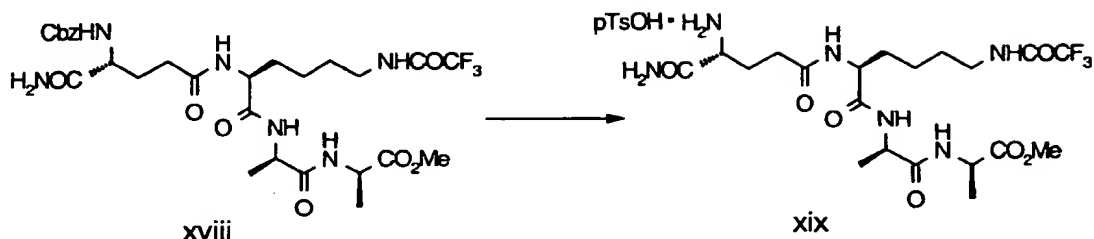
15 III. Preparation of Compound xviii



Trifluoroacetic acid (10 mL) is added to a solution of **compound xiv** (2.5 g, 5.0 mmol) in methylene chloride (10 mL) and the mixture is stirred for 2.5 h. The solution is concentrated *in vacuo* and dried under high vacuum to provide **compound xv**, the trifluoroacetate salt of **compound xiv**, as an oil. Separately, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.88g, 9.81 mmol) and N-hydroxysuccinimide (1.13 g, 9.81 mmol) are added to a solution of Cbz-D-iGln (2.20g, 7.85 mmol of **compound xvi**) in *N,N*-dimethylformamide (25 mL). The mixture is stirred for

3h, diluted to 100 mL with water and extracted with ethyl acetate (100 mL). The ethyl acetate solution is washed with saturated NaHCO_3 (100 mL), followed by brine (100 mL), and dried over sodium sulfate. The organic layer is evaporated
5 to give Cbz-D-iGln(NHS) (1.96 g, 5.19 mmol of **compound xvii**), the NHS-activated ester of **compound xvi**, as a white solid. The solid thus obtained is added to a solution of the above-prepared **compound xv** (2.60 g, 5.07 mmol) in anhydrous DMF (25 mL), after which *N,N*-diisopropylethylamine
10 (2.00 mL, 11.40 mmol) is added. The mixture is stirred for 72 h under N_2 . Water (200 mL) is added and the mixture is stirred for 2 h. The resulting solid is filtered, washed with water (50 mL), and dried in high vacuum to give **compound xviii** (2.20 g, 66% yield).

15 Analytical (**compound xviii**): mp 237-240°C; IR (KBr) 3302, 1699, 1669, 1630, 1184 cm^{-1} ; ^1H NMR(DMSO- d_6 , 300 MHz) δ 9.36(t, J = 5.0 Hz, 1H), 8.20(d, J = 7.0 Hz, 1H), 8.13(d, J = 7.7 Hz, 1H), 7.98(d, J = 7.3 Hz, 1H), 7.35(m, 5H), 7.29(brs, 1H), 7.00(brs, 1H), 5.01(s, 2H), 4.25(m, 3H),
20 3.90(m, 1H), 3.60(s, 3H), 3.15(q, J = 6.0, 12.0 Hz, 2H), 2.18(m, 2H), 1.85(m, 1H), 1.70(m, 1H), 1.55(m, 1H), 1.50(m, 3H), 1.28(d, J = 7.3 Hz, 3H), 1.27(m, 2H), 1.18(d, J = 7.3 Hz, 3H); MS (ESI) m/z 661 (100%, $M+H$); Anal. Calcd for $\text{C}_{28}\text{H}_{39}\text{N}_6\text{O}_9\text{F}_3$: C, 50.91; H, 5.95; N, 12.72. Found C, 51.13; H,
25 6.21; N, 12.47.

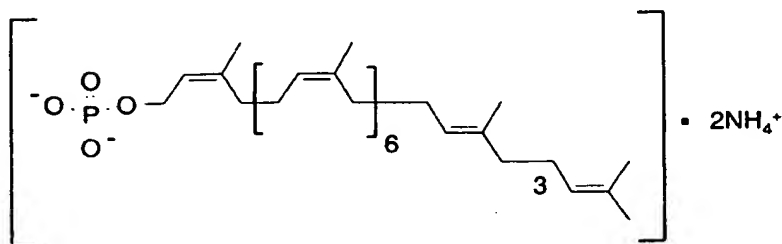
IV. Preparation of Compound xix

A mixture of **compound xviii** (2.15 g, 3.25 mmol), p-
 5 toluenesulfonic acid (662 mg, 3.25 mmol), and 5% Pd/C (500 mg) in methylene chloride:ethanol - 1:1 (100 mL) is hydrogenated at room temp for 1.5 h at 60 psi. The catalyst is filtered and the solution is evaporated to a foam. The foam is dried in high vacuum to provide homogeneous **compound**
 10 **xix** (2.20g, 97% yield).

Analytical (compound xix): IR(KBr) 3296, 1702, 1632, 1177 cm^{-1} ; ^1H NMR(DMSO- d_6 , 300 MHz), δ 9.37(t, J = 5.0 Hz, 1H), 8.23(d, J = 6.6 Hz, 1H), 8.14(m, 2H), 8.04(brs, 3H), 7.79(s, 1H), 7.60(s, 1H), 7.46(d, J = 8.0 Hz, 2H), 7.10(d, J = 8.0 Hz, 2H), 4.25(m, 3H), 3.72(t, J = 5.0 Hz, 1H), 3.61(s, 3H), 3.14(q, J = 6.0, 12.0 Hz, 2H), 2.28(s, 3H), 2.25(m, 2H), 1.94(q, J = 6.0, 12.0 Hz, 2H), 1.55(m, 4H), 1.28(d, J = 7.3 Hz, 3H), 1.24(m, 2H), 1.18(d, J = 7.09 Hz, 3H); MS (ESI) m/z 527 (100%, $M+H$). Anal. Calcd for $\text{C}_{27}\text{H}_{41}\text{N}_6\text{O}_{10}\text{F}_3\text{S} \cdot 0.5 \text{H}_2\text{O}$: C, 45.83; H, 5.93; N, 11.87. Found C, 45.56; H, 5.73; N, 11.60.

Preparation of the Undecaprenyl Phosphate Diammonium Salt

Starting Material (Compound xx)



5

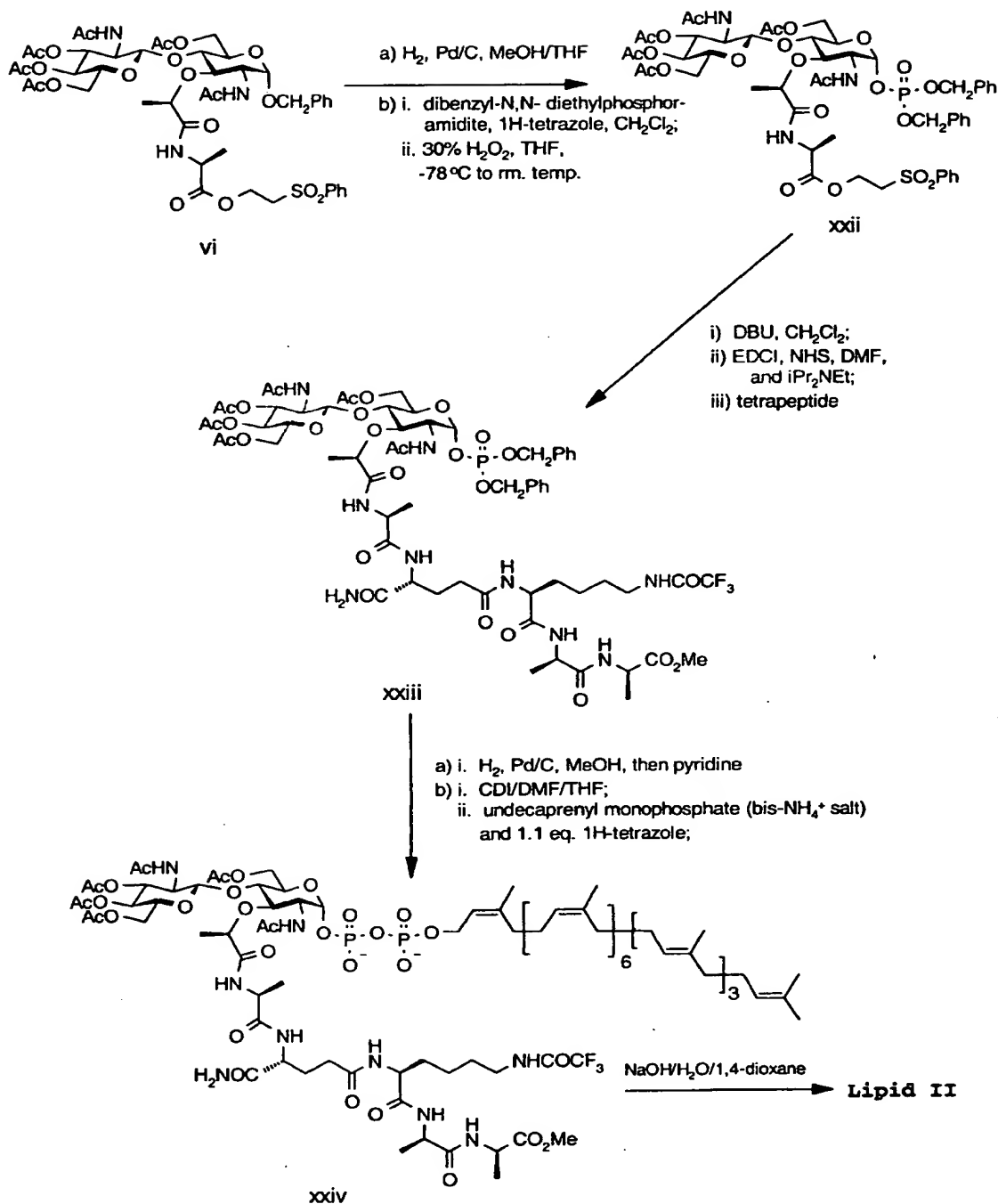
XX

Compound ~~xxx~~, undecaprenol (C₅₅) monophosphate, diammonium salt, is purchased from Polish Academy of Sciences, Warsaw, Poland.

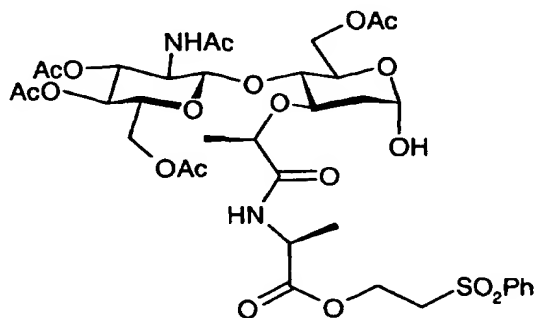
10

Preparation of Lipid II

A synthetic process for preparing lipid II, and key intermediates, from the starting material discussed above is outlined in Scheme III and exemplified below.



Scheme III

I. Preparation of the Lactol Intermediate (compound xxi)

xxi

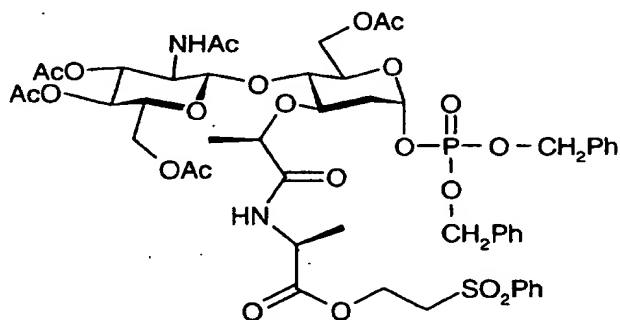
5 **Compound vi** (1.0 g, 1.0 mmol) is dissolved in 0.23 M HCl in acetic acid (5 mL) and added to a suspension of 10% Pd/C (0.50 g) in 0.23 M HCl in acetic acid (5 mL). The reaction mixture is stirred under an atmosphere of hydrogen (balloon, 15 psi) at 25 °C for 1.5 h. Analysis of the
10 reaction mixture by thin layer chromatography (5% MeOH/CHCl₃) shows complete consumption of starting material. The reaction mixture is diluted with EtOAc and the catalyst is collected by filtration through a pad of Celite. The filtrate is carefully washed with aq. NaHCO₃ (x3) and water
15 (x2). The aqueous extracts are combined and extracted with EtOAc. The combined organic layers are washed with brine, dried with Na₂SO₄, and concentrated *in vacuo* to afford the lactol product as a white solid (808 mg, 89% yield of **compound xxi**).

20 Analytical (compound xxi): ¹H NMR(DMSO-d₆) δ 1.02(d, J=6.84, 3H), 1.80(d, J=6.80, 3H), 1.70(s, 3H), 1.71(s, 3H),

1.84(s, 3H), 1.86(s, 3H), 1.89(s, 3H), 1.98(s, 3H), 3.30(m, 2H), 3.45(m, 2H), 3.6(m, 4H), 3.9(m, 4H), 4.2-4.4(m, 5H), 4.59(d, $J=8.31$, 1H), 4.81(t, $J=9.77$, 1H), 5.01(d, $J=2.93$, 1H), 5.12(t, $J=9.77$, 1H), 6.68(d, $J=4.4$, 1H), 7.60(m, 2H),
 5 7.70(m, 1H), 7.83(d, $J=8.31$, 2H), 8.02(d, $J=7.82$, 2H),
 8.23(d, $J=5.86$, 1H); ^{13}C NMR(DMSO- d_6 , 300 MHz) δ 16.71(q), 18.56(q), 20.24(q), 20.72(q), 22.57(q), 24.30(t), 30.36(q), 47.12(d), 53.67(t), 54.09(d), 58.09(t), 61.61(t), 62.45(t), 68.01(d), 68.33(d), 70.31(d), 72.37(d), 74.86(d), 75.76(d),
 10 76.73(d), 89.73(d), 89.54(d), 124.84(d), 127.68(d), 129.40(d), 133.98(d), 139.30(s), 169.25(s), 169.36(s), 169.42(s), 169.54(s), 169.89(s), 169.98(s), 171.52(s), 173.90(s). HRMS calcd for $\text{C}_{38}\text{H}_{54}\text{N}_3\text{O}_{20}\text{S}$, 904.3021, found 904.3011.

15

II. Preparation of the Monophosphate Triester Intermediate (compound xxii):



20

xxii

Compound ~~xxi~~ (1.4g, 1.55 mmol) in anhydrous dichloromethane (10 mL) is added rapidly via syringe to a vigorously stirred suspension of tetrazole (517 mg, 7.31

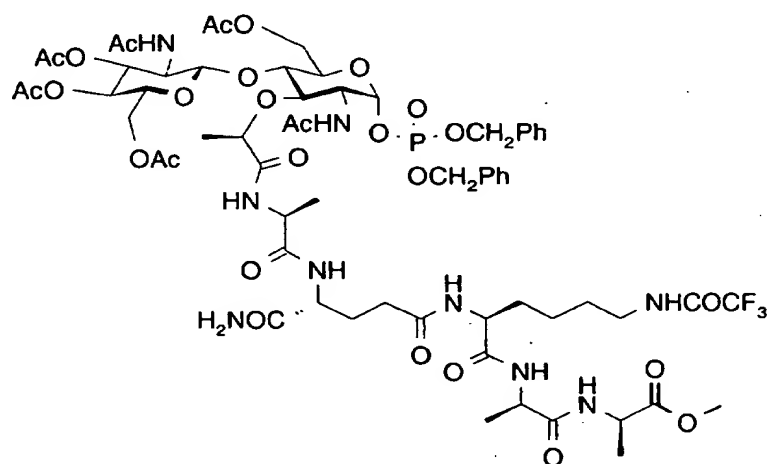
mmol) and dibenzyl *N,N'*-diethylphosphoramidite (1.4 mL, 4.70 mmol) in anhydrous dichloromethane (10 mL) under argon at 25°C. The reaction mixture becomes homogeneous within a few minutes. After 2 h, thin layer chromatography (10% MeOH/CHCl₃) shows a complete reaction. The mixture is diluted with dichloromethane (10 mL), then washed with saturated NaHCO₃ (5 mL), water (5mL) and brine (5 mL). The organic solution is dried over Na₂SO₄, and concentrated in vacuo to a colorless oil, which crystallized upon trituration with 1:1 diethyl ether/hexanes. The solids are filtered, dissolved in THF (30 mL), and cooled to -78 °C. Hydrogen peroxide (30%, 2.8 mL) is added dropwise via syringe to the vigorously stirred solution. After the addition is complete, the ice bath is removed and the mixture allowed to warm to room temperature over 2 h. TLC (4:1 EtOAc/acetone) shows complete reaction. The mixture is diluted with ice-cold saturated Na₂S₂O₃ (5 mL), followed by ethyl acetate (10 mL), and stirred for 5 minutes. The organic layer is dried over MgSO₄ and concentrated in vacuo to a colorless oil, which produced a white solid upon trituration with 1:1 Et₂O/hexanes. The white solid is dried under high vacuum at 40 °C to provide the monophosphate triester product (1.4g, 90% yield of **compound xxii**).

Analytical (compound xxii): ¹H NMR(DMSO-d₆, 500 MHz) δ
1.10(d, 3H), 1.20(d, 3H), 1.69(s, 3H), 1.75(s, 3H), 1.92(s,

-81-

3H), 1.96(s, 9H), 3.43(m, 1H), 3.57(m, 2H), 3.61(m, 1H),
 3.83(m, 4H), 4.05(m, 4H), 4.35(m, 3H), 4.60(m, 1H), 4.73(m,
 1H), 4.91(t, 1H), 5.00(m, 4H), 5.24(t, 1H), 5.81(m, 1H),
 7.35(m, 10H), 7.64(m, 2H), 7.74(m, 1H), 7.86(d, 2H), 8.06(d,
 1H), 8.39(d, 1H), 8.66(d, 1H); Anal. Calcd for:
 $C_{52}H_{66}N_3O_{23}PS \cdot 2 H_2O$: C, 52.04; H, 5.88; N, 3.50. Found C
 51.84; H, 5.73; N, 3.34. HRMS calcd for $C_{52}H_{66}N_3O_{23}PS$,
 1164.3624, found 1164.3601.

10 III. Preparation of the Disaccharyl Pentapeptide
Intermediate (compound xxiii)



xxiii

15 DBU (102 μ L, 0.68 mmol) is added dropwise to a solution
 of the monophosphate triester (793 mg, 0.68 mmol of **compound**
xxii) in dichloromethane (10 mL) under an argon atmosphere.
 After 15 minutes, thin layer chromatographic analysis (15%
 MeOH/ $CHCl_3$) shows complete consumption of the starting

material. The reaction solution is diluted with dichloromethane (20 mL) and washed with 1N HCl (30 mL). The organic layer is concentrated, then the resulting foam is triturated with Et₂O and allowed to stand at room temperature for about 2 h. After filtration, the crude acid product is dried at 40 °C for 2 h (465 mg, 68% yield).

The acid (465 mg, 0.47 mmol), N-hydroxysuccinimide (81 mg, 0.7 mmol) and EDCI (134 mg, 0.70 mmol) are dissolved in anhydrous DMF (4 mL) and allowed to stir for 18 h. Analysis of the reaction mixture by thin layer chromatography (15% MeOH/CHCl₃) reveals complete consumption of the starting material. The reaction mixture is concentrated in vacuo, redissolved in ethyl acetate (10 mL), then washed with water (10 mL) and brine (10 mL). The organic solution is dried with MgSO₄, and concentrated to a foam which, after trituration with diethyl ether (5 mL), provides the NHS-ester (398 mg, 78% yield).

The NHS-ester (398 mg, 0.34mmol), tetrapeptide (254 mg, 0.36mmol of **compound xix**) and iPr₂NEt (192 μL, 1.1 mmol) are dissolved in DMF (3 mL) and stirred at RT under argon for 18 h. The reaction mixture is then concentrated, dissolved in isopropanol/chloroform (1:9, 10 mL), washed with brine (3 X 5 mL), dried with MgSO₄ and concentrated in vacuo. The resulting oil is triturated with diethyl ether to provide

the disaccharyl pentapeptide **compound xxiii** (474 mg, 46% yield overall from **compound xxii**) as a white solid.

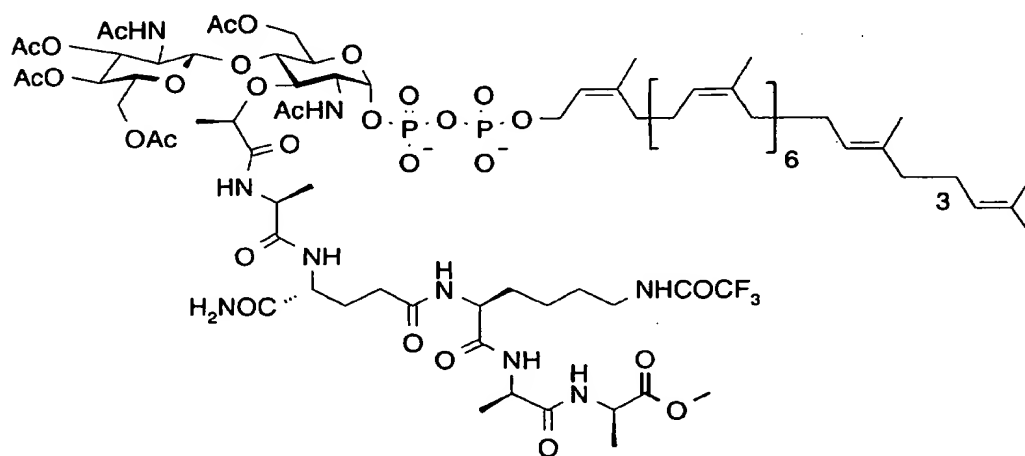
Analytical (compound xxiii): ^1H NMR(DMSO- d_6 , 500 MHz) δ
1.15-1.23(m, 12H), 1.40(m, 3H), 1.66(s, 3H), 1.69(s, 3H),
5 1.86(s, 3H), 1.89(s, 3H), 1.90(s, 3H), 1.91(s, 3H), 2.1(m,
2H), 3.1(m, 4H), 3.53(s, 3H), 3.71(m, 4H), 3.95(m, 3H), 4.1-
4.25(m, 10H), 4.34(t, $J=7.33$, 1H), 4.48(d, $J=6.84$, 1H),
4.67(d, $J=8.61$, 1H), 4.84(t, $J=9.77$, 1H), 4.98(m, 4H),
5.18(t, $J=9.77$, 1H), 5.57(m, 1H), 6.97(s, 1H), 7.25(s, 1H),
10 7.30(m, 10H), 7.97(m, 2H), 8.13(m, 3H), 8.15(d, $J=8.30$, 1H),
8.56(d, $J=5.37$, 1H), 9.32(m, 1H); Anal. Calculated for
 $\text{C}_{64}\text{H}_{89}\text{F}_3\text{N}_9\text{O}_{27}\text{P}\cdot 0.5 \text{ H}_2\text{O}$: C, 50.70; H, 5.99; N, 8.33. Found: C,
50.99; H, 6.38; N, 8.33. MS (ES $^-$) m/z 1503 (25%), 751.3
([M-2] $^{2-}$, 100%).

15 **Compound xxiii** can also be prepared by an alternative
procedure. To a solution of the free amine of the
tetrapeptide **compound xx** (0.068 g, 0.129 mmol) in CHCl_3 (1.3
mL) are added sequentially H_2O (1.3 mL), the acid of the
disaccharide (0.128 g, 0.129 mmol), and 1-
20 hydroxybenzotriazole (HOBt) (0.017 g, 0.129 mmol). The
mixture is then cooled to 0 $^\circ\text{C}$ and *N*-ethyl-*N'*-[3-
(dimethylamino)propyl]carbodiimide (EDCI) (0.027 g, 0.142
mmol) is added. The reaction is stirred vigorously for 2
days at 4 $^\circ\text{C}$. 1N HCl is added and the layers are
25 partitioned. The organic phase is washed with 1N HCl, H_2O ,

saturated NaHCO_3 , H_2O , and brine. The combined aqueous phases are extracted with CHCl_3 (x3). The combined organic phase is dried with Na_2SO_4 and concentrated *in vacuo* to an oil. The residue is purified by normal phase

5 chromatography over silica to yield **compound xxiii** (57%) as a colorless solid. This alternative procedure is described in Ho, G-J; Emerson, K. M.; Mathre, D. J.; Shuman, R. F.; Grabowski, D. J. "Carbodiimide-Mediated Amide Formation in a Two-Phase System. A High-Yield and Low-Racemization
10 Procedure for Peptide Synthesis," *J. Org. Chem.* **1995**, *60*, 3569-3570, incorporated herein by reference.

IV. Preparation of the Protected Lipid II Intermediate
(compound xxiv)



xxiv

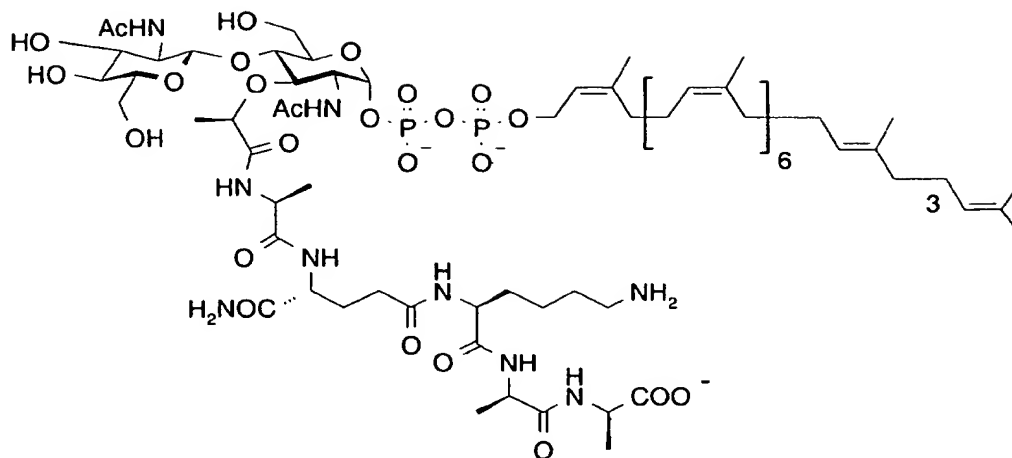
The disaccharyl pentapeptide (53 mg, 0.035 mmol of **compound xxiii**) is added to a suspension of 10% Pd/C (100 mg) in methanol (6.0 mL), cooled in an ice bath to aid in

degassing the reaction solution. The solution is then warmed to room temperature and hydrogenated at atmospheric pressure for 1.5 h. The catalyst is collected by filtration, and the resulting solution is treated with
5 pyridine (1.0 mL). The resulting mixture is concentrated in vacuo to an off-white solid, which is collected and dried under high vacuum for 16 h (ESI-MS m/z 1322, [M-H]).

The off-white solid is added to a mixture of 1,1'-carbonyldiimidazole (26.0 mg, 0.16 mmol) in anhydrous DMF
10 (2.5 mL) and anhydrous THF (2.0 mL). After stirring for 2 hours, the reaction is determined to be complete by mass spectroscopy (ESI-MS m/z 1372.4, [M-H]). Methanol (0.6 mL) is added, and the resulting solution stirred for 15 min. The mixture is then concentrated in vacuo and evaporated
15 from pyridine (1.0 mL). The solid material obtained, is dried under high vacuum for 2 h.

The residue is dissolved in DMF (0.4 mL) and dry THF (1.6 mL), to which a solution of undecaprenyl phosphate diammonium salt (21.4 mg, 0.024 mmol of **compound xx**) in THF
20 (0.5 mL) and DMF (0.1 mL) is added. 1H-tetrazole (1.8 mg, 0.03 mmol) is added and the mixture is stirred for 4 days under an argon atmosphere. Afterwards, the solution is concentrated in vacuo to give **compound xxiv**.

25 V. Preparation of Lipid II:



Lipid II

The residue from the previous step (**compound xxiv**) is dissolved in 1,4-dioxane:water (1:1; 2 mL). 1N sodium hydroxide (0.3 mL) is added and the resulting mixture stirred for 2 h. The reaction solution is filtered through an aqueous filter disc and purified by reverse phase HPLC on a Dynamax C8 100 Å, 5μ, 21.4 mm X 25 cm column using a gradient elution of 15:85 A:B to 0:100 A:B over 30 min, at a flow rate of 21.6 ml/min (where A = 0.05M aqueous ammonium bicarbonate and B = methanol). The retention time of the product is 18 min (detection at 214 nm). Lyophilization of the pure fractions gives 13.8 mg of Lipid II (24% yield overall from **compound xxiii**). Ultra High Resolution MS calcd for C₉₄H₁₅₇N₉O₂₅P₂, 1874.07659; found, 1874.08023.

Verification of Lipid II Structure

I. Biochemical Assay

The structural identity of Lipid II is validated via a biochemical assay employing a modified version of Lipid II bearing a dansyl tag on the ϵ -amino group of the L-lysine
5 residue. The substrate is prepared by direct dansylation of Lipid II in analogy to the conditions disclosed in Weppner, W.A., and F.C. Neuhaus, *J.Biol. Chem.*, **252**(7), 2296 (1977).

Weppner and Neuhaus had previously demonstrated the *in situ* generation of Lipid II by incorporating a Park
10 nucleotide bearing a dansyl tag on the L-lysine residue into glycan strands via incubation with a membrane fraction prepared from *Gaffkya homari*. In these experiments, the glycan strands were readily detected by descending paper chromatography followed by exposure of the chromatograms to
15 ultraviolet light. A similar experiment utilizing a membrane particulate fraction from *E. coli*. with a dansyl-tagged version of Lipid I (the immediate precursor to Lipid II that lacks the NAG carbohydrate subunit) has also been reported. See, Auger, G., et al., *Lett. Pept. Sci.*, **4**, 371
20 (1997).

In analogous fashion, incubation of our dansyl-tagged Lipid II derivative with a monofunctional transglycosylase (MtgA) from *Staphylococcus aureus* (described in U.S. Patent No. 5,922,540 and incorporated herein by reference) and
25 subsequent analysis of the reaction mixtures by thin layer

chromatography, under the conditions reported by Weppner and Neuhaus, results in the visualization of glycan strands upon exposure to ultraviolet light in exact analogy to the precedent cited above. (See figure)

5 Although a dansyl tag was used in the experiments described above, other tags well-known to those skilled in the art can also be used. As used herein, "tag" refers to an element or compound containing atoms that can be distinguished from their normal counterparts by physical
10 means (e.g., radioactivity assay, mass spectrography or fluorescence), and can thus be used to follow (trace) the metabolism of the normal substances.

II. Protocol for Biochemical Transglycosylation

15 In the figure, the following protocols are used for the corresponding lanes represented in the thin layer chromatography (TLC) strip.

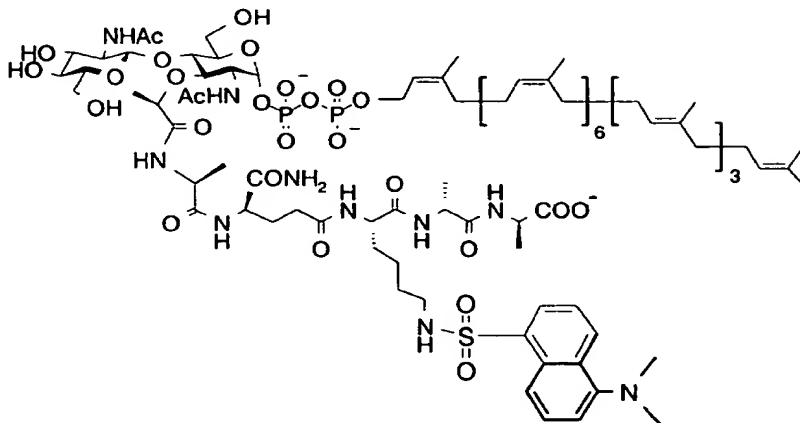
20 Lane 1: 5 mL substrate stock solution (300 mM dansyl lipid II in 75 mM PIPES at pH 6.1, 37.5 mM MgCl₂)

 Lanes 2 and 3: Lane 1 plus 5 mL MtgA enzyme solution (10 mM stock in 25 mM HEPES at pH 7.5, 150 mM NaCl, 0.5% CHAPS); incubate 20 min @ 25 °C; stop with 30 mL methanol at 0 °C.

25 Lane 4: Add 1 mL of lysozyme stock (1mg/mL hen eggwhite lysozyme, HEWL)

Location of reaction products is visualized by exposing the TLC to UV light (366 nm). (See Figure)

III. Preparation of Dansylated Lipid II:



5

Lipid II with dansyl tag

A solution of dansyl chloride (13.8 mg, 5.1 μmol) in acetone (250 μL) is added in one portion to a solution of Lipid II (2.4 mg, 1.3 μmol) in 0.25M aq NaHCO_3 (250 μL).

10 The mixture, which turns turbid immediately, is stirred at room temperature for 90 minutes. Electrospray MS and HPLC show complete conversion to product. The mixture is concentrated *in vacuo*, redissolved in 1:1 water/acetonitrile and purified by preparative chromatography on a Dynamax C8

15 column (100Å, 5 μ , 21.4 mm x 25 cm) using a gradient of 85:15 to 100:0 methanol/50mM ammonium bicarbonate over 20 minutes (detection at 214 nm). The appropriate fractions are combined and lyophilized to provide dansylated Lipid II (1.7

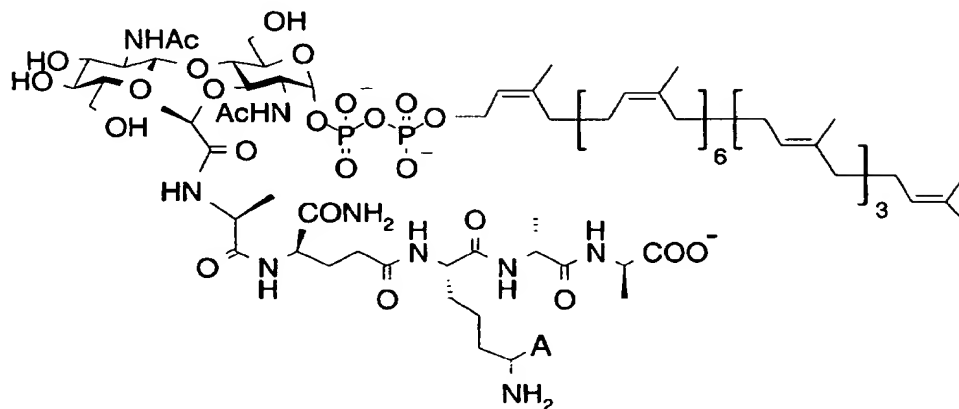
X-13168

-90-

mg, 63% yield). Ultra High Resolution MS calcd for
 $C_{106}H_{168}N_{10}O_{27}P_2S$, 2107.12764; found, 2107.12725.

WE CLAIM:

1. A non-radioactive, isolated, Lipid II compound of the following formula:



5

3W⁺

wherein:

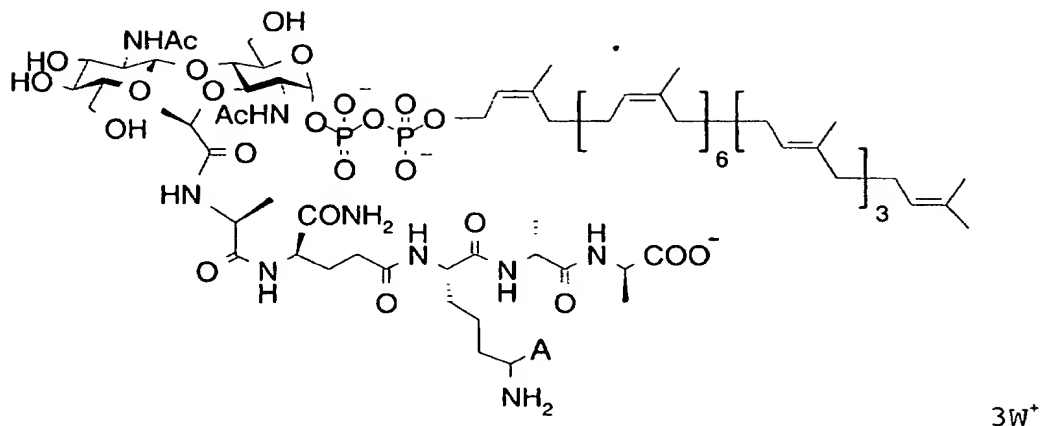
A is a hydrogen or a carboxyl group;

Ac is -C(O)CH₃; and

W⁺ is each independently a proton or cation selected from the group consisting of an alkali metal, alkaline earth metal, ammonium, alkyl ammonium, and dialkyl ammonium.

10

2. An isolated Lipid II compound having a purity greater than or equal to 50% of the following formula:



wherein:

A is a hydrogen or a carboxyl group;

Ac is $-C(O)CH_3$; and

5 W⁺ is each independently a proton or cation selected from the group consisting of an alkali metal, alkaline earth metal, ammonium, alkyl ammonium, and dialkyl ammonium.

3. The isolated Lipid II compound of Claims 1 or 2,
10 wherein said Lipid II compound has a purity greater than or equal to 60%.

4. The isolated Lipid II compound of Claims 1 or 2,
15 wherein said Lipid II compound has a purity greater than or equal to 70%.

5. The isolated Lipid II compound of Claims 1 or 2
wherein said Lipid II compound has a purity greater than or equal to 80%.

6. The isolated Lipid II compound of Claims 1 or 2, wherein said Lipid II compound has a purity greater than or equal to 90%.

5

7. The isolated Lipid II compound of Claims 1 or 2, wherein said Lipid II compound has a purity greater than or equal to 95%.

10 8. The isolated Lipid II compound of Claims 1 or 2, wherein said Lipid II compound has a purity greater than or equal to 98%.

9. The isolated Lipid II compound of Claims 1 or 2,
15 wherein said Lipid II compound has a purity greater than or equal to 99%.

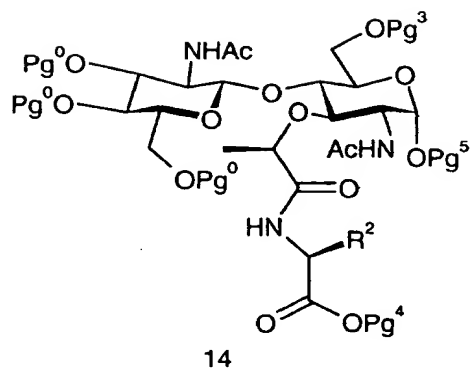
10. The isolated Lipid II compound of Claims 1 or 2
wherein said Lipid II compound has a purity greater than or
20 equal to 99.5%.

11. A process for preparing a Lipid II compound,
comprising:

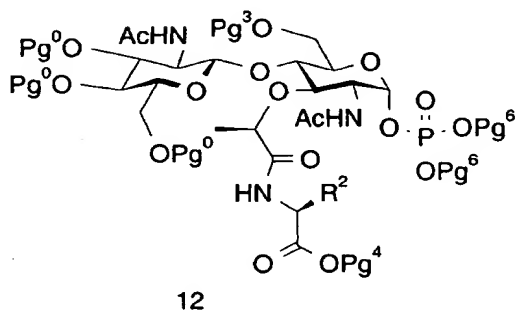
(1) providing a protected disaccharide core of formula

25 14

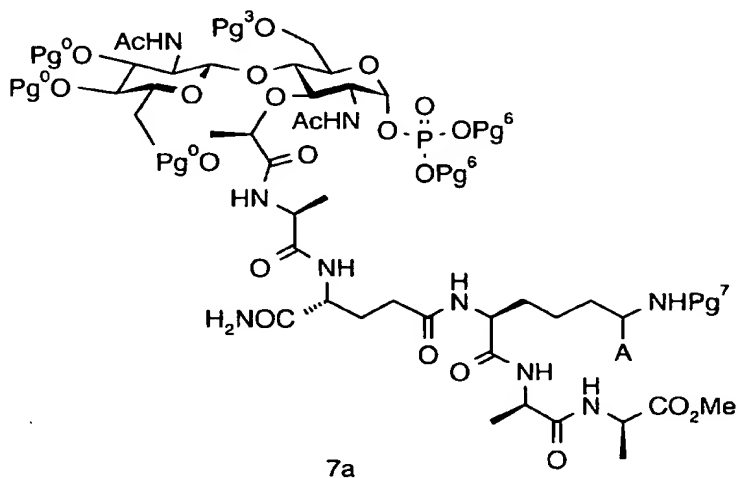
-94-



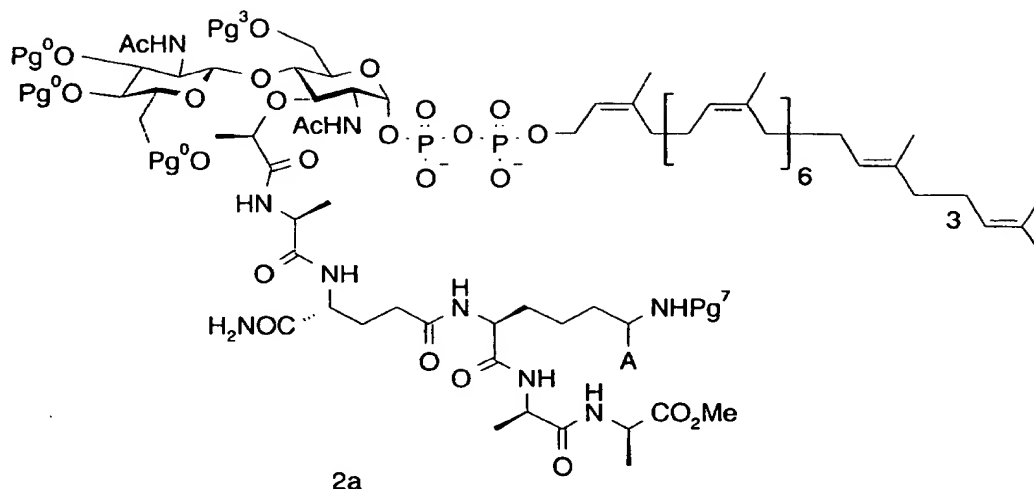
(2) introducing an anomeric phosphate to form a compound of formula 12



5 (3) introducing a polypeptide linkage to form a compound of formula 7a



(4) introducing an undecaprenyl diphosphate linkage to form a compound of formula 8a



(5) removing Pg^0 , Pg^3 , Pg^7 , and Pg^8 to form said Lipid II compound;

wherein:

5 A is hydrogen or a carboxyl group;
R² is methyl;
Ac is -C(O)CH₃;
Pg⁰ is an acyl hydroxy-protecting group;
Pg³ is an acyl hydroxy-protecting group;
10 Pg⁴ is a carboxy-protecting group;
Pg⁵ is a hydroxy-protecting group;
Pg⁶ is a phosphate protecting group;
Pg⁷ is an amine-protecting group; and
Pg⁸ is a carboxy-protecting group.

15

12. A Lipid II compound prepared by the process of Claim 11.

13. A process for isolating Lipid II comprising isolating said Lipid II at a pH greater than 6.

14. The process of Claim 13 wherein said pH is between
5 6 and 12.

15. The process of Claim 14 wherein said pH is between 7 and 10.

10 16. The process of Claim 15 wherein said pH is between 7 and 9.

15 17. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 50%.

18. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 60%.

20 19. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 70%.

20. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 80%.

25 21. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 90%.

22. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 95%.

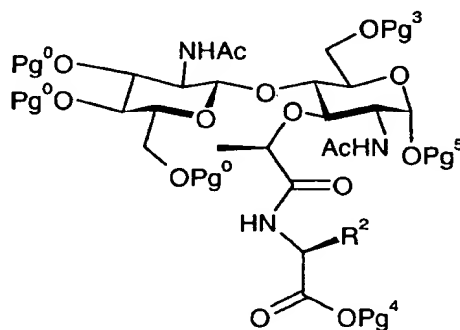
5 23. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 98%.

24. The process of Claims 13, 14, 15 or 16, wherein said Lipid II has a purity greater than or equal to 99%.

10

25. A process for preparing a Lipid substrate, comprising:

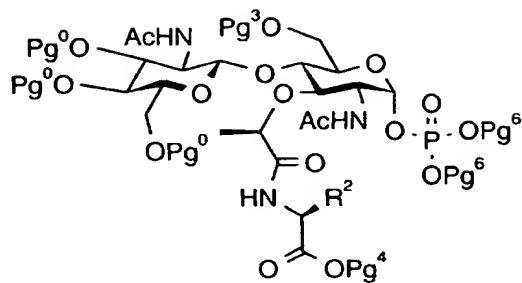
(1) providing a protected disaccharide of formula 14



14

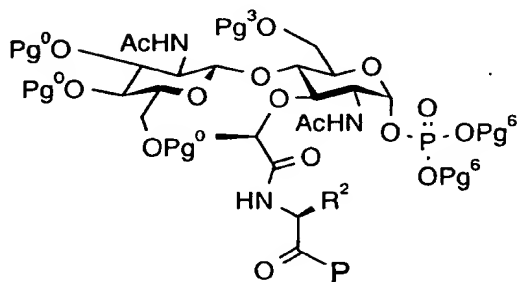
15 (2) introducing an anomeric phosphate to form a compound of formula 12

-98-



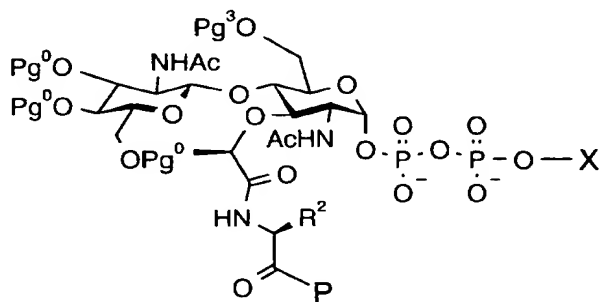
12

(3) introducing a peptide linkage to form a compound of formula 7



7

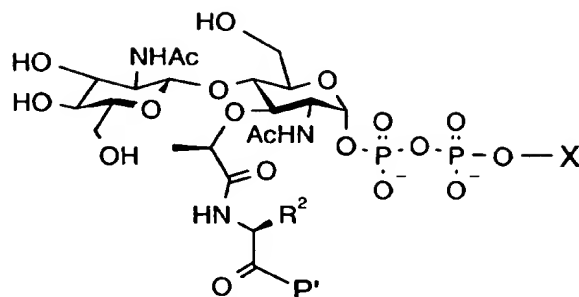
5 (4) introducing a lipid-carrier diphosphate linkage to form a compound of formula 2



2

(5) removing the Pg⁰ and Pg³ groups and deprotecting the P group to produce a lipid substrate of formula 1

-99-



1

wherein:

Ac is $-\text{C}(\text{O})\text{CH}_3$;

Pg^0 is an acyl hydroxy-protecting group;

5 Pg^3 is an acyl hydroxy-protecting group;

Pg^4 is a carboxy-protecting group;

Pg^5 is a hydroxy-protecting group;

Pg^6 is a phosphate-protecting group;

R^2 is hydrogen, $(\text{C}_1\text{-C}_5)$ alkyl or $(\text{C}_1\text{-C}_3)$

10 alkylphenyl;

X is a lipid carrier;

P attached to the carbonyl is a residue of an amino acid or peptide, wherein P comprises a protected terminal carboxy group; and

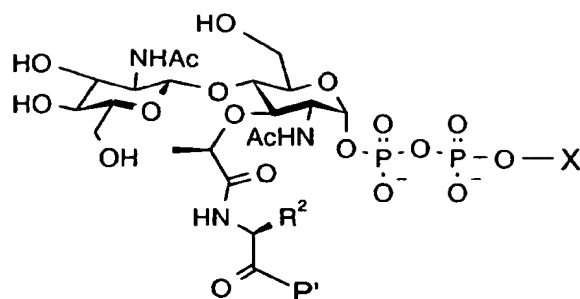
15 P' is a residue of an amino acid or peptide.

26. A Lipid substrate prepared by the process of Claim

25.

20 27. A lipid II analog of formula 1

-100-



1

wherein:

Ac is $-\text{C}(\text{O})\text{CH}_3$;

Pg^0 is an acyl hydroxy-protecting group;

5 Pg^3 is an acyl hydroxy-protecting group;

Pg^4 is a carboxy-protecting group;

Pg^5 is a hydroxy-protecting group;

Pg^6 is a phosphate-protecting group;

R^2 is hydrogen, $(\text{C}_1\text{-C}_5)$ alkyl or $(\text{C}_1\text{-C}_3)$

10 alkylphenyl;

X is a lipid carrier;

P attached to the carbonyl is a residue of an amino acid or peptide, wherein P comprises a protected terminal carboxy group; and

15 P' is a residue of an amino acid or peptide.

-101-

ABSTRACT

A process is described for preparing a substrate for the transglycosylase enzymes of bacterial cell wall biosynthesis. The chemical synthesis makes available a
5 sustainable and substantially pure source of supply of lipid II, including analogs thereof, that may be used in the identification of new therapeutic agents capable of disrupting steps in bacterial cell wall biosynthesis.

TRANSGLYCOSYLATION PROCESS

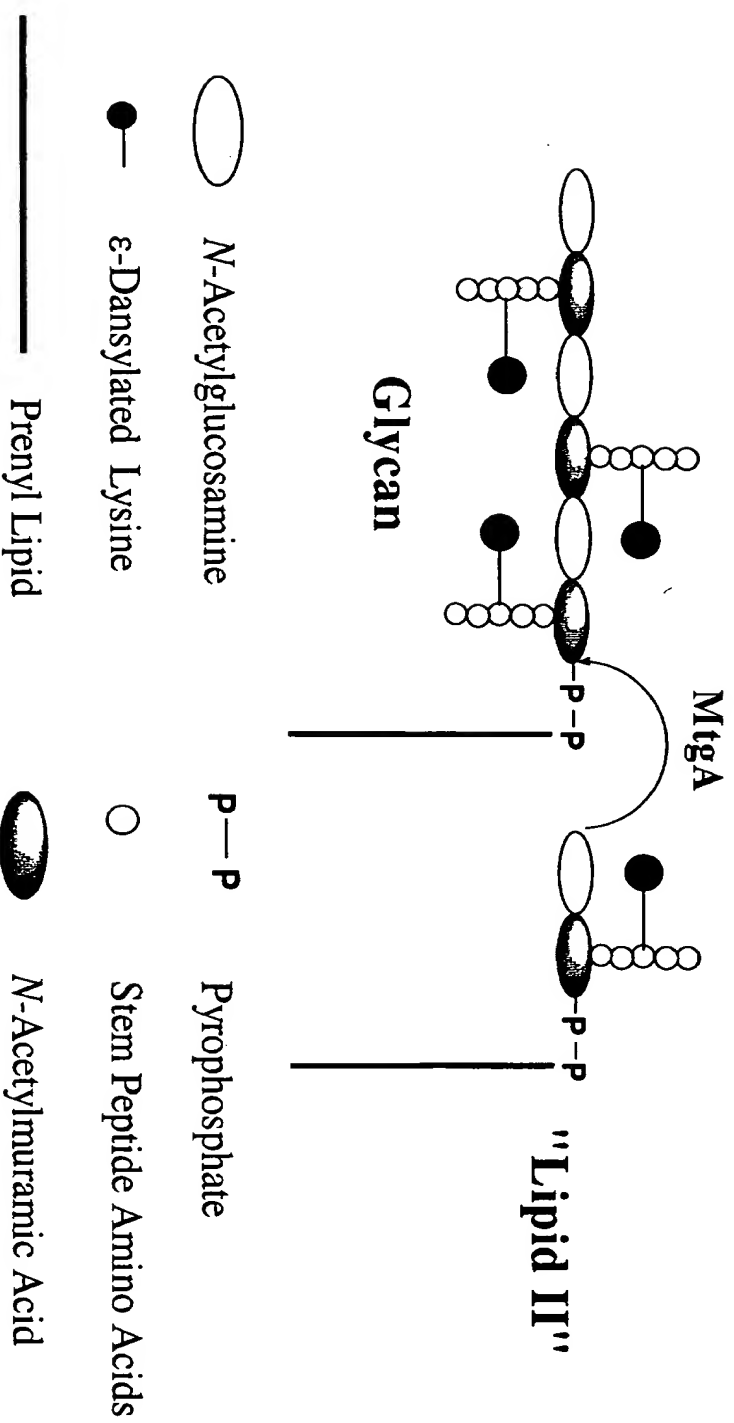
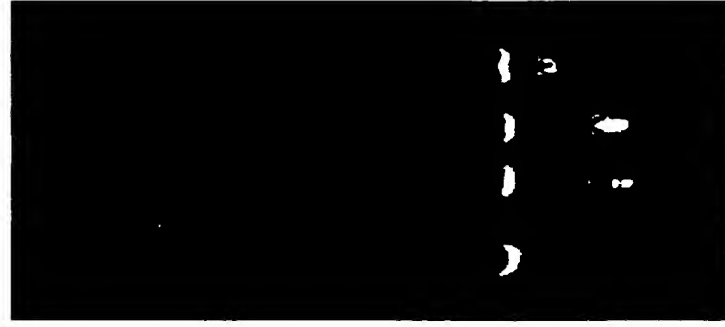


FIG. 1

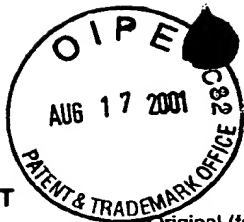
FLUORESCENCE DETECTION OF GLYCAN



Lane 1: Dansyl Lipid II
Lane 2: Dansyl Lipid II + MtgA
Lane 3: Dansyl Lipid II + MtgA
Lane 4: Lysozyme Digest

1 2 3 4

FIG. 2



Petition under 37 CFR 1.183
Attachment #2
Copy of Request Form for
PCT/US01/12637 as filed on
18 April 2001

1/5

PCT REQUEST

X-13168

Original (for SUBMISSION) - printed on 18.04.2001 05:17:39 PM

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form - PCT/RO/101 PCT Request	
0-4-1	Prepared using	PCT-EASY Version 2.91 (updated 01.01.2001)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	Applicant's or agent's file reference	X-13168
I	Title of Invention	PROCESS FOR PREPARING LIPID II
II	Applicant	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States
II-4	Name	ELI LILLY AND COMPANY
II-5	Address:	Lilly Corporate Center Indianapolis, IN 46285 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	317.433.4983
II-9	Facsimile No.	317.276.3861
III-1	Applicant and/or Inventor	
III-1-1	This person is:	inventor only
III-1-4	Name (LAST, First)	ALBORN, William, Ernest, Junior
III-1-5	Address:	1320 Thistlewood Court Carmel, IN 46032 United States of America
III-2	Applicant and/or Inventor	
III-2-1	This person is:	inventor only
III-2-4	Name (LAST, First)	BLASZCZAK, Larry, Chris
III-2-5	Address:	1327 Broadway Indianapolis, IN 46202 United States of America

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III-3	Applicant and/or Inventor	
III-3-1	This person is:	inventor only
III-3-4	Name (LAST, First)	MAULDIN, Scott, Carl
III-3-5	Address:	5540 Snowberry Court Indianapolis, IN 46221 United States of America
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III-4-4	Name (LAST, First)	SKATRUD, Paul, Luther
III-4-5	Address:	5233 Tony Drive Greenwood, IN 46143 United States of America
III-5	Applicant and/or Inventor	
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III-5-4	Name (LAST, First)	VANNIEUWENHZE, Michael, Scott
III-5-5	Address:	8259 Glengarry Court Indianapolis, IN 46236 United States of America
III-6	Applicant and/or Inventor	
III-6-1	This person is:	inventor only
III-6-4	Name (LAST, First)	ZIA-EBRAHIMI, Mohammad, Sadegh
III-6-5	Address:	7724 Cove street Indianapolis, IN 46254 United States of America
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	COHEN, Charles, E.
IV-1-2	Address:	ELI LILLY AND COMPANY Lilly Corporate Center Indianapolis, IN 46285 United States of America
IV-1-3	Telephone No.	317.433.4983
IV-1-4	Facsimile No.	317.276.3861
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	PARKER, Raymond, S., III

PCT REQUEST

X-13168

Original (for SUBMISSION) - printed on 18.04.2001 05:17:39 PM

V	Designation of States	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	<p>AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT</p> <p>EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT</p> <p>EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR and any other State which is a Contracting State of the European Patent Convention and of the PCT</p> <p>OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT</p>
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	<p>AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CR CU CZ DE DK DM DZ EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG UZ VN YU ZA ZW</p>
V-3	National Patent (States which have become party to the PCT after the issuance of this version of EASY)	CO
V-5	<p>Precautionary Designation Statement</p> <p>In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.</p>	
V-6	Exclusion(s) from precautionary designations	NONE
VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	18 April 2000 (18.04.2000)
VI-1-2	Number	60/198,000
VI-1-3	Country	US
VI-2	<p>Priority document request</p> <p>The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):</p>	VI-1

PCT REQUEST

X-13168

Original (for SUBMISSION) - printed on 18.04.2001 05:17:39 PM

VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)	
VIII	Check list	number of sheets	electronic file(s) attached
VIII-1	Request	5	-
VIII-2	Description	90	-
VIII-3	Claims	10	-
VIII-4	Abstract	1	EZABST00.TXT
VIII-5	Drawings	2	-
VIII-7	TOTAL	108	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-9	Separate signed power of attorney	✓	-
VIII-10	Copy of general power of attorney	reference no. X-13168	-
VIII-16	PCT-EASY diskette	-	diskette
VIII-17	Other (specified):	Return Receipt Postcard	-
VIII-17	Other (specified):	Delegation of Authority	-
VIII-17	Other (specified):	PCT International Application Cover Letter	-
VIII-18	Figure of the drawings which should accompany the abstract	1	
VIII-19	Language of filing of the international application	English	
IX-1	Signature of applicant or agent	<i>Charles E. Cohen</i>	
IX-1-1	Name	ELI LILLY AND COMPANY	
IX-1-2	Name of signatory	Cohen, Charles E.	
IX-1-3	Capacity	Attorney	

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

PCT REQUEST

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FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
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1/2

PCT (ANNEX - FEE CALCULATION SHEET)

X-13168

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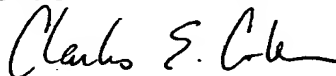
(This sheet is not part of and does not count as a sheet of the international application)

0	For receiving Office use only	
0-1	International Application No.	
0-2	Date stamp of the receiving Office	
0-4	Form - PCT/RO/101 (Annex)	
0-4-1	PCT Fee Calculation Sheet Prepared using	PCT-EASY Version 2.91 (updated 01.01.2001)
0-9	Applicant's or agent's file reference	X-13168
2	Applicant	ELI LILLY AND COMPANY
12	Calculation of prescribed fees	fee amount/multiplier total amounts (USD)
12-1	Transmittal fee T	⇒ 240
12-2	Search fee S	⇒ 846
12-3	International fee	
	Basic fee	
	(first 30 sheets) b1	382
12-4	Remaining sheets	78
12-5	Additional amount (X)	9
12-6	Total additional amount b2	702
12-7	b1 + b2 = B	1,084
12-8	Designation fees	
	Number of designations contained in international application	87
12-9	Number of designation fees payable (maximum 6)	6
12-10	Amount of designation fee (X)	82
12-11	Total designation fees D	492
12-12	PCT-EASY fee reduction R	-117
12-13	Total international fee (B+D-R) I	⇒ 1,459
12-14	Fee for priority document	
	Number of priority documents requested	1
12-15	Fee per document (X)	15
12-16	Total priority document fee P	⇒ 15
12-17	TOTAL FEES PAYABLE (T+S+I+P)	⇒ 2,560
12-19	Mode of payment	authorization to charge deposit account
12-20	Deposit account instructions	
	The receiving Office:	United States Patent and Trademark Office (USPTO) (RO/US)
12-20-1	Is hereby authorized to charge the total fees indicated above to my deposit account	✓
12-20-2	Is hereby authorized to charge any deficiency or credit any over-payment in the total fees indicated above to my deposit account	✓
12-20-3	Is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account	✓

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12-21	Deposit account No.	05-0840
12-22	Date	18 April 2001 (18.04.2001)
12-23	Name and signature	Charles E. Cohen 

VALIDATION LOG AND REMARKS

13-2-2	Validation messages States	Green? More designations could be made. The following States have not been designated: US. Please verify.
		Yellow! Additional national designation added: Obtain updated maintenance tables rather than using this field.
13-2-8	Validation messages Payment	Green? Please ensure that you have a valid deposit account with the receiving Office selected.